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(54) Title: D-ENZYME COMPOSITIONS AND METHODS OF THEIR USE

(57) Abstract

D-enzyme compositions are described comprising an amino acid residue sequence that defines a polypeptide able to catalyze an enzymatic reaction. The D-enzyme has an amino acid residue sequence consisting essentially of D-amino acids.

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D-ENZYME COMPOSITIONS AND
METHODS OF THEIR USE

Technical Field

5 The present invention relates to proteins incorporating D-amino acid residues. More particularly, the present invention relates to enzymically active proteins consisting essential of D-amino acids and methods for using such proteins.

10

Background

15 The biosphere is inherently chiral; each class of biological macromolecules is made up of monomer molecules of uniform chirality (Mason, Chirality 3:223, 1991) and the biochemical interactions of biological macromolecules are inherently chiral.

20 Enzymes, for example, invariably act only on one enantiomer of a chiral substrate, or generate only one diastereomer from a prochiral substrate. Fersht, in "Enzyme Structure and Mechanism", W.H. Freeman and Company, San Francisco, 1977, pp. 75-81. This specificity can be related to the chiral structure of the enzyme molecule, including the three-dimensional folding of the polypeptide backbone and the 25 orientation of the amino acid side chains in the folded protein molecule. Fersht, supra. To date only L-enzymes have been described in nature; this leaves the description of D-enzymes and their properties, which include folded structure, enzymatic 30 activity, and chiral specificity, as unexplored questions.

35 Recently, Zawadzke et al., J. Am. Chem. Soc., 114:4002-4003, 1992, described the preparation of a small 45 amino acid residue polypeptide (D-rubrodoxin) using D-amino acids. L-rubrodoxin is found in clostridia and is the simplest iron-sulfur protein. It is believed to function in electron

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transport. However, it lacks an demonstrated enzymic activity.

5 Prior to the present invention, the largest L-protein known to be chemically synthesized in a conventional step-wise fashion is Preprogonadotropin Release Hormone (PreproGnRH). PreproGnRH has 93 amino acid residues. (Milton et al., *Biochemistry*, (1992) 31: 8800.) PreproGnRH inhibits prolactin release.

10 Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L or R and S are used to denote the absolute configuration 15 of the molecule about its chiral center(s). The prefixes (+) and (-) or d and l are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or l meaning that the compound is levorotatory. A compound prefixed with 20 (+) or d is dextrorotatory. For a given chemical structure, these compounds, called stereoisomers, are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such 25 isomers is often called an enantiomeric or racemic mixture.

30 The property of optical activity is due to molecular asymmetry about carbon atoms that are linked to four different atoms or molecules. Where there is only one asymmetric carbon atom, or chiral center as it is sometimes called, there are two possible stereoisomers. Where there are n asymmetric carbons or chiral centers, the number of potential stereoisomers increases to 2^n . Thus, a molecule with 35 three chiral centers would have eight possible stereoisomers.

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While the structural differences between stereoisomers are subtle and of little consequence in ordinary chemical reactions, they may be profound where biological systems are concerned, i.e., if the 5 compounds are utilized in enzyme-catalyzed reactions. Thus, the L-amino acids are readily metabolized in humans but the corresponding D-analogs are not, and only D-glucose can be phosphorylated and processed into glycogen or degraded by the glycolytic and 10 oxidative pathways of intermediary metabolism. Similarly, beta blockers, pheromones, prostaglandins, steroids, flavoring and fragrance agents, pharmaceuticals, pesticides, herbicides, and many other compounds exhibit critical stereospecificity. 15 In the field of pesticides, Tessier [Chemistry and Industry, Mar. 19, 1984, p. 199] has shown that only two of the eight stereoisomers of deltamethrin, a pyrethroid insecticide, have any biological activity. The same statement concerning the concentration of 20 bioactivity in a single isomer can be made about many other pesticides, including the phenoxypropionates and halopropionate derivatives, each containing one chiral center and existing in the form of two optical isomers.

25 Stereochemical purity is of equal importance in the field of pharmaceuticals, where 12 of the 20 most prescribed drugs exhibit chirality. A case in point is provided by naproxen, or (+)-S-2-(6-methoxy-2-naphthyl)-propionic acid, which is one of the two 30 most important members of a class of 2-aryl-propionic acids with non-steroidal anti-inflammatory activity used, for instance, in the management of arthritis. In this case, the S(+) enantiomer of the drug is known to be 28 times more therapeutically potent than 35 its R(-) counterpart. Still another example of chiral pharmaceuticals is provided by the family of

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beta-blockers, the L-form of propranolol is known to be 100 times more potent than the D-enantiomer.

Synthesis of chiral compounds by standard organic synthetic techniques generally leads to a 5 racemic mixture which, in the aggregate, may have a relatively low specific bioactivity since certain of the stereoisomers in the mixture are likely to be biologically or functionally inactive. As a result, larger quantities of the material must be used to 10 obtain an effective dose, and manufacturing costs are increased due to the co-production of stereochemically "incorrect" and hence, inactive ingredients.

In some instances, certain isomers may actually 15 be deleterious rather than simply inert. For example, the D-enantiomer of thalidomide was a safe and effective sedative when prescribed for the control of morning sickness during pregnancy. However, its L-thalidomide counterpart was discovered 20 to be a potent mutagen.

Methods are available for stereoselective synthesis that generally involve chemical synthesis and isolation steps that are lengthy, complex and costly. Moreover, a synthetic scheme capable of 25 producing one specific enantiomer cannot be applied in a general way to obtain other optically active compounds. What is needed is a generalized approach to the resolution of racemic mixtures produced by ordinary chemical reactions, and a number of 30 approaches have been used.

A widely used approach has been the selective precipitation of desired compounds from racemic mixtures. See, for example, Yoshioka et al. [U.S. Pat. No. 3,879,451], Paven et al. [U.S. Pat. No. 35 4,257,976], Halmos [U.S. Pat. No. 4,151,198], and Kameswaran [U.S. Pat. No. 4,454,344].

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The above procedures successfully resolved racemic mixtures because treatment of the mixtures with optically pure reagents produced diastereomers which, unlike the initial racemic compounds, have 5 different physical properties. Thus, fractional crystallization or other physical means may be employed to separate diastereomeric compounds.

Separation of diastereomers can also be carried out by chromatography. For example, Pollock et al. 10 [J. Gas Chromatogr. 3: 174 (1965)] have resolved diastereomeric amino acids by gas chromatography. Mikes et al. [J. Chromatogr. 112:205 (1976)] have used liquid chromatography to resolve diastereomeric dipeptides. In most cases, the optically pure 15 reagents have been in the stationary phase during chromatographic separation, but they may also be used in elutants. Hare et al. [U.S. Pat. No. 4,290,893] have used liquid chromatography to resolve racemic mixtures that were treated with aqueous elutants 20 containing optically pure reagents and metal cations; resolution occurred because the resulting diastereomeric complexes had different partition coefficients in the chromatographic system.

All of the methods described to this point have 25 relied upon the availability of suitable optically pure reagents, but such reagents are often not available or else their use is prohibitively expensive. In an alternative approach, enzymatic resolution techniques have been developed. Many 30 different classes of enzymes have been used for the resolution of stereoisomers on a preparative scale, including hydrolases (especially the lipases and esterases such as chymotrypsin), lyases, and oxidoreductases (e.g., amino acid oxidases and 35 alcohol reductases). Generally speaking, enzymes for use in resolutions should ideally exhibit broad substrate specificity, so that they will be capable

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of catalyzing reactions of a wide range of "unnatural" substrates, and a high degree of stereoselectivity for catalyzing the reaction of one isomer to the exclusion of others.

5 The hydrolases (e.g., lipases and esterases) are among the more attractive enzymes for use in resolutions, because they do not require expensive cofactors, and some of them exhibit reasonable tolerance to organic solvents. Additionally, chiral
10 chemistry often involves alcohols, carboxylic acids, esters, amides, and amines with chiral carbons, and carboxyl hydrolases are preferred choices as stereoselective catalysts for reactions of such species. For instance, enzymatic treatment has been
15 applied to the resolution of racemic mixtures of amino acid esters. Stauffer [U.S. Pat. No. 3,963,573] and Bauer [U.S. Pat No. 4,262,092].

20 Separation of reaction products from enzymes has been facilitated by attaching the enzyme to a solid support which could be removed by centrifugation or packed into a column through which the racemic mixtures were passed.

25 Enzymes have also been explored for the resolution of classes of compounds other than the amino acids discussed above. Immobilized lipase in principal resolves mixtures by enzymatic hydrolysis or transesterification. In the case of a biphasic hydrolysis reaction, the differing solubility properties of the acids and esters involved required
30 the dispersion and agitation of mixtures containing the immobilized solid-phase enzyme, an aqueous buffer, and the water-immiscible organic phase containing solvent and reactant--a relatively inefficient process.

35 Enzymes have been applied to the resolution of optical isomers of insecticides. For instance, Mitsuda et al. [Eur. Patent Appl'n. Publ. No. 0 080

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827 A2] contacted a racemic acetic acid ester with stereoselective esterases of microbial and animal origin in biphasic systems (i.e., aqueous/organic dispersion). In related work on optically purified 5 pyrethroids, Mitsuda et al. [U.S. Pat. No. 4,607,013] employed microbial esterases. Klibanov et al. [U.S. Pat. No. 4,601,987] resolved racemic 2-halopropionic acids by means of lipase-catalyzed esterification reactions conducted in organic media.

10 Additional examples can also be provided of the state-of-the-art enzyme-mediated resolution as applied to the production of optically purified pharmaceuticals. Sih [U.S. Pat. No. 4,584,270] has disclosed enzymatic means for the production of 15 optically pure (R)-4-amino-3-hydroxybutyric acid, a key intermediate in the preparation of L-carnitine.

20 Until recently only naturally occurring L-enzymes could be described, and this left the presumed properties of D-enzymes, including their folded structures, enzymatic activity and chiral specificity, as unexplored questions. What was needed was sufficient progress in the chemical synthesis of proteins to make possible the total 25 synthesis of the D-enantiomer of whole enzymes in sufficient quantity to form crystals and to perform other functions.

Brief Summary of the Invention

A new type of enzyme designated a D-enzyme has 30 been discovered that has ability to catalyze the reaction of a chiral substrate. Therefore, described herein is a D-enzyme comprising an amino acid residue sequence that defines an polypeptide able to catalyze an enzymatic reaction, wherein the amino acid residue sequence consists essentially of D-amino acids.

35 The enzymatic reaction can have achiral substrate specificity, or chiral substrate specificity. Preferred achiral substrate-specific D-

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enzymes are superoxide dismutase or carbonic anhydrase. A preferred chiral substrate-specific D-enzymes is HIV-1 protease.

The invention also contemplates a method of
5 producing a chirally pure chemical comprising:

a) reacting in an aqueous admixture a first stereoisomer substrate with a D-enzyme that specifically converts the first stereoisomer substrate into a chiral reaction product, wherein the
10 reaction occurs for a time period and under reaction conditions sufficient to form a reaction product; and

b) isolating the chiral reaction product from the admixture, thereby forming the chirally pure
15 chemical. In alternative embodiments, the aqueous admixture may comprise a racemic mixture or partial racemic mixture of a substrate having at least a first and a second stereoisomer or may comprise the first stereoisomer of the substrate alone.

20 A D-enzyme of this invention provides a wide variety of benefits and advantages which are apparent to the skilled practitioner. A D-enzyme provides a means to efficiently produce chirally pure chemicals for use as reagent grade industrial chemicals, and as
25 pharmaceutically pure medicaments. In addition, a D-enzyme can be used in combination with its L-enzyme counterpart in co-crystallization admixtures to form racemic crystals for determining crystallographic structures using X-ray diffraction data.

30 Furthermore, because of the inherent resistance of a D-enzyme from proteolysis by natural L-amino acid-specific proteases, therapeutically administered D-enzymes that have achiral substrate specificity can be utilized in place of the corresponding L-enzyme
35 and enjoy prolonged half-lives in proteolytic environments such as the blood or digestive tract,

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thereby increasing the effectiveness of the therapeutic enzyme.

A D-enzyme of the present invention may also be employed for screening natural product libraries.

5 More particularly, a D-enzyme may be employed to identify chiral inhibitors within a natural product library. In some instance, a natural product library may include a chiral inhibitor having activity with respect to D-enzyme but having no 10 activity with respect to the corresponding L-enzyme. Synthesis of the enantiomer of the identified chiral inhibitor then results in the formation of an inhibitor of the corresponding L-enzyme.

15 Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

20 Figure 1 illustrates the molecular weight characterization of the D- and L-enzyme enantiomers of the HIV-1 protease as described in Example 2 using reconstructed ion spray mass spectroscopy. The molecular weight is expressed in daltons, and is shown as a peak of the percent (%) of relative intensity of the measured spectra. Figure 1A 25 illustrates molecular weight data obtained with the L-enzyme, and Figure 1B illustrates data obtained with the D-enzyme.

Figure 2 illustrates the comparative enzyme 30 activity of the HIV PR enzyme D- and L-enantiomers on D- and L-enantiomers of a chiral fluorogenic substrate as described in Example 3. Figure 2A shows L-enzyme with L-substrate; Figure 2B shows L-enzyme with D-substrate; Figure 2C shows D-enzyme with L-substrate; and Figure 2D shows D-enzyme with 35 D-substrate. Data is expressed as activity, measured in arbitrary units of fluorescence intensity, over a reaction time course in minutes.

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Figure 3 illustrates ribbon representations of the polypeptide backbone of the homodimeric HIV-1 protease molecule in both L- and D- conformations, shown in the left and right portions of the Figure, respectively. The arrows indicate the direction of the polypeptide in the amino- to carboxy-terminus direction.

Figure 4 illustrates a schematic representation of the chemical segment ligation strategy employed for the total synthesis of D- and L-[Aba⁶⁷⁻⁹⁵(CO-S)⁵¹⁻⁵²]₂, HIV-1 protease analogs.

Figure 5 illustrates a schematic representation of the optimized solid-phase chain assembly tactics employed in the synthesis of the functionalized peptide segments. Deprotection and coupling reactions are separated by a single flow wash step.

Figure 6 illustrates a composite chromatogram showing two purified functionalized unprotected D-[α COSH]HIV-1 PR(1-51) and D-[N^a-BrCH₂CO]HIV-1(53-99) segments and the final (48 hour) D-[Aba⁶⁷⁻⁹⁵(CO-S)⁵¹⁻⁵²]₂, HIV-1 PR ligation product (bold) run on a Vydac 218TP5415 column eluted by gradient (40-55% B), at a flow rate of 1 milliliter per minute.

Figure 7 illustrates the step reaction yields for the synthesis of the D- and L-[Aba⁶⁷⁻⁹⁵(CO-S)⁵¹⁻⁵²]₂, HIV-1 protease analogs.

Figure 8 illustrates the ion spray mass spectra of the HPLC purified [(NHCH₂COSCH₂CO)⁵¹⁻⁵²]HIV-1 PR monomers.

Figure 9 illustrates the reverse phase HPLC measurements of D- & L-[Aba⁶⁷⁻⁹⁵, (CO-S)⁵¹⁻⁵²]₂ HIV-1 PR ligation products.

Figure 10 illustrates the far-ultraviolet circular dichroism spectrum of the D- and L-[Aba⁶⁷⁻⁹⁵(CO-S)⁵¹⁻⁵²]₂, HIV-1 protease analogs.

Figure 11 illustrates the enzymatic activity of the [Aba⁶⁷⁻⁹⁵, (CO-S)⁵¹⁻⁵²]₂, HIV-1 PR enantiomers on D- and

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L-isomers of the substrate Ac-Thr-Ile-Nle-Nle-Gln-
Arg.amide.

5 Detailed Description of the Invention

A. Definitions

10 Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are either in the "L" or "D" stereoisomeric form. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 C.F.R. 1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

20 TABLE OF CORRESPONDENCE

	<u>SYMBOL</u>	<u>AMINO ACID</u>
	<u>1-Letter</u>	<u>3-Letter</u>
	Y	Tyr
	G	Gly
25	F	Phe
	M	Met
	A	Ala
	S	Ser
	I	Ile
	L	Leu
30	T	Thr
	V	Val
	P	Pro
	K	Lys
35	H	His
	Q	Gln
	E	Glu

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	Z	Glx	Glu and/or Gln
	W	Trp	tryptophan
	R	Arg	arginine
	D	Asp	aspartic acid
5	N	Asn	asparagine
	B	Asx	Asn and/or Asp
	C	Cys	cysteine
	J	Xaa	Unknown or other

10 The above symbols are employed for both L- and D-
amino acid residues. The symbol Xaa is employed for
any unknown or other amino acid residue. However,
the symbol Xaa is frequently employed herein to
designate L- or D- α -amino-n-butyric acid (aba), an
15 isosteric replacement for Cys residues.

It should be noted that all amino acid residue
sequences represented herein by formulae have a left-
to-right orientation in the conventional direction of
amino terminus to carboxy terminus. In addition, the
20 phrase "amino acid residue" is broadly defined to
include the amino acids listed in the Table of
Correspondence and modified and unusual amino acids,
such as those listed in 37 C.F.R. 1.822(b)(4), and
incorporated herein by reference. Furthermore, it
25 should be noted that a dash at the beginning or end
of an amino acid residue sequence indicates a peptide
bond to a further sequence of one or more amino acid
residues or a covalent bond to an amino-terminal
group such as NH₂ or acetyl or to a carboxy-terminal
30 group such as COOH.

35 Racemic Mixture: A racemic mixture is used herein
to refer to a mixture of at least a first and second
stereoisomer in any proportions. In this context,
the term "resolution" as used herein will refer to
separation of a first racemic mixture into second and
third mixtures wherein the proportions of the two
stereoisomers in the second and third mixtures are

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different from that in the first racemic mixture, the proportion being greater in one and necessarily smaller in the other.

5 B. D-Enzyme Compositions

The present invention contemplates a D-enzyme comprising a molecule having an amino acid residue sequence that defines a polypeptide able to catalyze an enzymatic reaction. A D-enzyme has an amino acid residue sequence consisting essentially of D-amino acids.

10 The term "D-amino acid" does not indicate the direction of specific rotation of the molecule because it is well known that some amino acids are dextrorotatory whereas others are levorotatory. Rather, the terms denotes an absolute configuration by convention relative to the two possible stereoisomers of glyceraldehyde, D-glyceraldehyde and L-glyceraldehyde. See for example, Lehninger, in 15 "Biochemistry", Worth Publishers, Inc., New York, 1970, pp. 76-78. Thus all stereoisomers that are stereochemically related to L-glyceraldehyde are designated L-, and those related to D-glyceraldehyde are designated D-, regardless of the direction of 20 rotation of plane polarized light given by the 25 isomer.

In the case of threonine and isoleucine, there are two stereochemical centers, i.e. the amino acid C α atoms and the C β atoms. The D-threonine and D-isoleucine employed herein have stereochemistries at both the amino acid C α atoms opposite to the stereochemistry of L-threonine and L-isoleucine, i.e. D-threonine and D-isoleucine are complete mirror 30 images of L-threonine and L-isoleucine, respectively.

35 Glycine is the only commonly occurring achiral amino acid. Accordingly, when a protein or enzyme is designated herein as a D- or L-protein or enzyme, it

is meant that essentially all of the chiral amino acid residue comprising such protein or enzyme have the indicated chirality. The presence of achiral amino acid residues such as glycine within a protein or enzyme does not affect the designation of its chirality, as employed herein.

All chiral amino acids in protein described in nature are L-amino acids. Thus, proteins having only D-configuration chiral amino acids in their amino acid residue sequence (referred to as D-proteins) are unknown in nature.

In one embodiment, it is preferred that a D-enzyme have an amino acid residue sequence that corresponds, and preferably is identical to, the amino acid residue sequence of a known or "natural" enzyme. By "natural" is meant a sequence present on an enzyme isolated from nature without laboratory-mediated interventions directed at altering the enzyme's sequence. By "known" is meant either a natural enzyme or an enzyme that is the product of a sequence modifying process that alters the amino acid sequence to produce an enzyme with known enzymatic properties.

Many enzymes described in the scientific literature, too numerous to recite here, have been the subject of mutation of their natural amino acid residue sequence such that they no longer correspond in amino acid residue sequence to the sequence of a natural isolate, and yet still retain an enzymatic activity. Thus, in another embodiment, the invention contemplates D-enzymes having amino acid residue sequences that correspond to known enzymes.

A D-enzyme can have any of a variety of enzymatic activities as that activity is generally understood in biochemistry, meaning broadly the ability to reduce the activation energy of a reaction between one or more substrates to form one or more reaction products. For the purposes of this invention, it is

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useful to distinguish enzyme substrate specificities that are chiral and achiral.

Chiral specificity refers to the selectivity of an enzyme to catalyze the reaction of only one of two stereoisomers. Achiral specificity refers to the ability of the enzyme to react with a substrate that does not present a recognition-dependent asymmetric structure to the enzyme, i.e., enzyme-substrate recognition and catalysis is not dependent upon the presence of an asymmetric structure in the substrate binding region of the enzyme. Stated differently and in the context of the present invention relating to enantiomeric selectivity of a D-enzyme, an achiral substrate can be catalyzed by either a D- or L-enzyme because no asymmetric structures are present in the achiral substrate upon which enzyme binding and catalysis depends. In contrast, a chiral substrate can only be catalyzed by one or the other of a D- and L-enzyme pair because structural asymmetry of the substrate is involved in the binding and catalysis.

A further distinction can be made between chiral and achiral reaction products. For example, an achiral substrate may be converted into a chiral or an achiral reaction product. If an achiral substrate is converted to a chiral reaction product, the chirality of the reaction product will depend upon the chirality of the enzyme, i.e. an L- or D-enzyme. Similarly, a chiral substrate may be converted into a chiral or an achiral reaction product.

Many enzymes exhibit chiral specificity including the preferred and exemplary enzyme, HIV-1 protease. Similarly, there are many enzymes that exhibit achiral specificity, including superoxide dismutase and carbonic anhydrase.

Thus in one embodiment, the invention contemplates a D-enzyme having chiral specificity that converts (catalyzes the reaction of) a chiral substrate into a

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reaction product, but does not also convert the enantiomer (stereoisomer) of the chiral substrate. An example is the HIV-1 protease described herein which reacts only with the D-substrate and not the L-substrate.

In another embodiment, the invention contemplates a D-enzyme having achiral specificity wherein both the D-enzyme and the corresponding L-enzyme convert an achiral substrate into a reaction product. One example is the reaction catalyzed by superoxide dismutase upon superoxide radicals. Another example is the reaction catalyzed by carbonic anhydrase.

A D-enzyme of this invention can be any size (length of amino acids), and can be comprised of multiple subunits, as is well known for many characterized enzymes. A multiple subunit D-enzyme is comprised of all D-protein subunits. Protein subunits that make up an enzyme, or single protein subunit enzyme, range widely in size. Typical enzyme subunits are from 80 to 500 amino acid residues in length, although shorter and longer proteins are known, from about 50 amino acid residues to sizes in excess of 4000 amino acid residues.

The present invention in one embodiment generally concerns the use of D-enzymes in processes for the stereoselective synthesis or resolution of racemic mixtures of chiral organic acids, alcohols, amines, esters, amides, nitriles, hydantoins, and other chiral compounds in which an enzyme is used that is capable of stereoselectively catalyzing a reaction to convert one isomer of a chiral precursor to a chemically distinct optically active compound.

Enzymes are well suited to the role of stereoselective catalysis inasmuch as they contain asymmetric, catalytically active sites in which the molecule being synthesized or undergoing resolution may bind. Because these enzyme active sites are

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themselves asymmetric, they permit two enantiomers of a given racemic substrate to be acted upon differentially, and they permit chiral products to be formed from achiral precursors.

5 For example, many enzymes exist that effectively catalyze the hydrolysis or condensation of ester and amide chemical functional groups. Many of these enzymes, but not all of them, belong to either one of two main classes of enzymes known as hydrolases or
10 lyases as defined in the Recommendations of the Commission on Biochemical Nomenclature, Elsevier, Amsterdam, The Nomenclature and Classification of Enzymes (1972) p. 17-22. The term E.C. followed by a series of numbers as used herein, provides the
15 identification of an enzyme pursuant to the Commission Recommendations.

20 Types of enzymes useful in the practice of the present invention include, but are not limited to, enzymes that catalyze the following categories of reactions:

hydrolysis of esters to form acids and alcohols;
formation of esters (i.e., esterifications) from acids and alcohols;
transesterification, i.e., reaction of an ester
25 with an alcohol or acid to form a different ester and a different alcohol or acid;
transaminations (e.g., reaction between an alpha-keto acid and an amino acid);
hydrolysis of amides (including peptide bonds and
30 N-acyl compounds) to form acids and amines;
formation of amides (including peptides) from acids and amines (or amino acids);
hydrolysis of amino acid hydantoins to yield carbamoyl amino acids and amino acids; and
35 hydrolysis of nitriles to form the corresponding amides and carboxylic acids (and in particular,

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hydrolysis of amino nitriles to amino amides and amino acids).

Specific examples of such enzymes include but are not limited to trypsin, chymotrypsin, thermolysin, 5 rennin, pepsin, papain, carboxy peptidases, amino peptidases, penicillin and cephalosporin acylase, acetyl cholinesterase, cholesterol esterase, and mammalian pancreatic lipases and peptidases

Preferred esterases include chymotrypsin (E.C. 10 3.4.21.1) because of its high stereoselectivity, and broad substrate range. Other esterases include, but are not limited to, carboxyl esterase (E.C. 3.1.1.1.), carboxypeptidase A (E.C. 3.4.17.1), acetyl cholinesterase (E.C. 3.1.1.7), pepsin (E.C. 15 3.4.23.1), trypsin (E.C. 3.4.21.4) and papain (E.C. 3.4.22.2).

Amino acid residue sequences for natural enzymes, and published modified enzymes, useful for the present invention are generally available in the 20 published literature and on computer data bases.

Preferred and widely used protein sequence data bases include Geneseq™, GenBank®, EMBL, Swiss-Prot, PIR and GenPept, all of which are commercially available from Intelligenetics, Inc. (Mountain View, California).

25 The complete three-dimensional structure for many enzymes suitable for use in this invention are available from the Brookhaven Protein Data Bank, Brookhaven National Laboratories, Upton, NY. Exemplary proteins with their respective Protein Data 30 Bank Codes (PDB numbers) that are included in the data base include:

(1hvp): hiv-1 protease complex with substrate;
(2hvp): hiv-1 protease; (3hvp): (aba==67,95==)-hiv-1 protease,sf2 isolate; (4hvp): hiv-1 protease complex 35 with the inhibitor n-acetyl-*thr-*ile-*nle-psi(ch2-nh)-*nle-*gln-*arg amide (mvt-101) (SEQ ID NO 1); (2cyp): cytochrome c peroxidase (e.c.1.11.1.5);

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(1gp1): glutathione peroxidase (e.c.1.11.1.9);
(4cat): catalase (e.c.1.11.1.6);
(7cat): catalase (e.c.1.11.1.6); (8cat): catalase
(e.c.1.11.1.6); and (2sod): cu,zn superoxide
5 dismutase (e.c.1.15.1.1).

Particularly preferred are the antioxidant enzymes of the superoxide dismutase (SOD) class. Because of the wide distribution of SOD enzymes in aerobic organisms, many isolates of SOD have been reported in many species. A recent literature search revealed descriptions of the sequence of 26 different SOD enzymes in mammals, non-mammals, bacteria, yeast and plants including human EC-SOD, [Hjalmarsson et al., Proc. Natl. Acad. Sci. USA, 84:6340-6344 (1987)];
10 human SOD [Sherman et al., Natl. Acad. Sci. USA, 80:5465-5469], and Schneider et al., Cell, 54:363-368 (1988); bovine SOD [Steinman et al., J. Biol. Chem., 249:7326-7338, (1974)]; equine SOD [Lerch et al., J. Biol. Chem., 256:11545-11551 (1981)]; murine SOD
15 [Getzoff et al., Proteins: Struct. Func. Genet., 5:322-336 (1989)]; porcine SOD [Schinina et al., FEBS Lett., 186:267-270 (1985)]; rabbit SOD [Reinecke et al., Biol. Chem., 369:715-725 (1988)]; ovine SOD [Schinina et al., FEBS Lett., 207:7-10 (1986)]; rat
20 SOD [Steffens et al., Z. Physiol. Chem., 367:1017-1024 (1986)]; drosophila SOD [Nucleic Acids Res., 17:2133-2133 (1989)]; xenopus SOD [Eur. J. Biochem. (1989)]; brucella SOD [Beck et al., Biochemistry, 29:372-376 (1990)]; caulobacter SOD [Steinman et al.,
25 J. Bacteriol. (1988)]; neurospora SOD [Lerch, J. Biol. Chem., 260:9559-9566 (1985)]; photobacterium SOD
30 [Steffens et al., Z. Physiol. Chem., 364:675-690 (1983)]; schistosoma SOD [Simorda et al., Exp. Parasitol., 67:73-84 (1988)]; yeast SOD [Steinman et al., J. Biol. Chem., 255:6758-6765 (1980)];
35 cauliflower SOD [Steffens et al., Biol. Chem. Hoppe-

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Seyler, 367:1007-1016 (1986)]; cabbage SOD [Steffens et al., Physiol. Chem., 367:1007-1016 (1986)]; maize SOD [Cannon et al., Proc. Natl. Acad. Sci. USA, 84:179-183 (1987)]; pea SOD [Scioli et al., Proc. Natl. Acad. Sci. USA, 85:7661-7665 (1988)]; spinach SOD [Kitagawa et al., J. Biochem., 99:1289-1298 (1986)]; and tomato SOD [Plant Mol. Biol., 11:609-623 (1988)]. Any of these varieties of SOD are suitable for use as a D-enzyme of the present invention.

Carbonic anhydrase C is another preferred enzyme suitable for the preparation of a D-enzyme. Carbonic anhydrase C catalyses the reaction that combines carbon dioxide and water to form bicarbonate and hydrogen ions. The sequence of carbonic anhydrase C is described by Henderson et al., Biochim. Biophys. Res. Comm., 52:1388 (1973); Lin et al., J. Biol. Chem., 249:2329 (1974).

Other optically specific enzymes that react with a chiral substrate and are therefore useful as a D-enzyme of this invention have been extensively described in United States Patent Nos. 5,077,217, 5,057,427, 4,800,162, and 4,795,704, which are specifically incorporated herein by reference.

C. Synthesis of a D-Enzyme

A D-enzyme of the present invention can be prepared by any means available to one skilled in the polypeptide arts. The precise method employed for synthesizing the polypeptide is not considered essential to the basic structure of a D-enzyme of this invention, and therefore is not to be considered as limiting, particularly as technology develops new ways to synthesize and assemble polypeptides.

Preferred routes of polypeptide synthesis include:

- 35 1. Conventional chemical synthesis, e.g. step wise synthesis, and

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2. Assembly of polypeptide building blocks by
chemical ligation.

It is presently considered impractical to employ conventional chemical (step-wise) synthetic methods 5 to produce polypeptides having more than 100 amino acid residues. On the other hand, chemical ligation methods may be employed to assemble polypeptides several time larger. Accordingly, for D-enzymes greater than 100 amino acid residues, chemical or 10 enzymatic ligation techniques are presently the only practical means for making such products. Described herein is a ligation strategy for preparing 10-100 milligram amounts of the D- and L-HIV-1 protease enzymes.

15 Although not presently available, manufactured protein synthesis apparatus using D-proteins may solve the problem of incorporating D-amino acids in the protein translation machinery, making it possible to synthesize D-enzymes using recombinant DNA expression 20 of messenger RNA and D-amino acids.

Conventional Step-wise Syntheses:

Synthetic chemistry techniques, such as the stepwise addition of amino acids in a solid-phase Merrifield-type synthesis, are preferred for reasons 25 of purity, antigenic specificity, freedom from undesired side products, ease of production and the like. An excellent summary of the many techniques available for synthesizing L-proteins and enzymes can be found in Steward et al., in "Solid Phase Peptide 30 Synthesis", W.H. Freeman Co., San Francisco, 1969; Bodanszky et al., in "Peptide Synthesis", John Wiley & Sons, Second Edition, 1976 and Meienhofer, in "Hormonal Proteins and Peptides", Vol. 2, p. 46, Academic Press (New York), 1983; and Kent, Ann. Rev. 35 Biochem., 57:957, 1988, for solid phase peptide synthesis, and Schroder et al., in "The Peptides", Vol. 1, Academic Press (New York), 1965 for classical

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solution synthesis, each of which is incorporated herein by reference. Appropriate protective groups usable in such synthesis are described in the above texts and by McOmie, in "Protective Groups in Organic 5 Chemistry", Plenum Press, New York, 1973, which is incorporated herein by reference.

In general, the solid-phase synthesis methods contemplated comprise the sequential addition of one or more amino acid residues or suitably protected 10 amino acid residues to a growing peptide chain.

Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized 15 for amino acids containing a reactive side group such as lysine.

For the synthesis of a D-enzyme, D-amino acids or protected D-amino acids are utilized rather than the conventional L-amino acids. D-amino acids suitable 20 for polypeptide synthesis are commercially available from the Peptide Institute (Osaka, Japan); Peptides International (Louisville, KY); Bachem Bioscience (Philadelphia, PA); and Bachem California, (Torrance, CA).

Using a solid phase synthesis as exemplary, the 25 protected or derivatized D-amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next D-amino acid in the sequence having the 30 complimentary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The 35 protecting group of the amino or carboxyl group is then removed from this newly added D-amino acid residue, and the next D-amino acid (suitably

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protected) is then added, and so forth. After all
the desired D-amino acids have been linked in the
proper sequence, any remaining terminal and side
group protecting groups (and solid support) are
5 removed sequentially or concurrently, to afford the
final polypeptide.

Ligation Techniques:

The chemical ligation of polypeptides has recently
been described by Kent in United States Application
10 Serial No. 07/865,368, filed April 8, 1992, whose
disclosures are hereby incorporated by reference.
This technique is preferred for D-enzymes having a
length of 100 amino acid residues or greater. In this
procedure, two polypeptides are first synthesized,
15 and contain termini adapted for chemical ligation.
After stepwise chemical synthesis and cleavage from
their respective solid phase resins, the two
polypeptides are mixed and reacted to join the
adapted termini and form a larger, linear polypeptide
20 comprised of the two polypeptides.

An exemplary step-wise synthesis of a D-enzyme is
detailed in Example 1 describing the synthesis of a
variety of D-HIV-1 protease. Example 5 discloses an
exemplary ligation-type synthesis of D- and L-[Aba⁶⁷.
25 ⁹⁵(CO-S)⁵¹⁻⁵²]₂HIV-1 protease analogs. The D-[Aba^{67,95}(CO-
S)⁵¹⁻⁵²]₂HIV-1 protease analog of Examaple 5 is
functionally equivalent to the D-HIV-1 protease of
Example 1.

Similar synthesis can be applied to the
30 preparation of D-superoxide dismutase, carbonic
anhydrase, or any of the other D-enzymes described
herein.

35 D. Methods for Screen Chemical Libraries

The most common means for identifying
pharmaceutically useful compounds involves screening

chemical libraries. The thoroughness such screening may be markedly enhanced by employing both L-enzymes and D-enzymes.

Natural product libraries isolated from nature may 5 consist of several hundred thousand compounds. Chemical libraries may also be prepared by chiral synthesis of particular enantiomers or by non-chiral synthesis of racemates. In the search for new drug candidates, such libraries may be screened to 10 identify compounds active in a particular assay. In many instances, the target molecule is an enzyme or an enzyme system and the search is directed to identifying drug candidates which can serve as specific inhibitors or cofactors of such enzyme or 15 enzyme system. Once a candidate compound is identified as an inhibitor of a specific therapeutically relevant enzyme, analogues of such candidate compound may be designed and synthesized so to improve its activity and other desirable 20 properties.

In many instances, chiral specificity is a necessary attribute of active substrates, cofactor, and inhibitors. However, it is disclosed herein that the chiral specificity of substrates, cofactors, and 25 inhibitors depends upon the chirality of the target enzyme. Accordingly, the target enzyme can often distinguish between active and inactive enantiomers of a given a substrate, cofactor, and inhibitor. Component elements of a natural product library often 30 display random chirality and bear to inherent relationship to the target enzyme. Accordingly, if only a single enantiomer is present within a library, the chirality of such enantiomer is as likely to be the wrong (inactive) enantiomer as to be the right 35 (active) with respect to any given target enzyme. If the library is screened against only the native (L)- configuration of a target enzyme, and if the elements

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of the library are non-racemate, i.e. if they are chiral, there is a significant chance (50/50) that the library include only the inactive enantiomer.

The present invention teaches that screening a
5 natural product library against both an L-enzyme and its corresponding D-enzyme, can approximately double the number of candidate compounds identified from such library.

Any candidate compound identified as active
10 against a D-enzyme may be inactive with respect to the corresponding L-enzyme. However, structural analysis of a candidate compound active with respect to a D-enzyme is predictive of the structure of a candidate compound active with respect to the
15 corresponding L-enzyme, i.e. the enantiomers of compounds found to be active with respect to a D-enzyme are likely to have corresponding activity with respect to the corresponding L-enzyme.

Accordingly, the number of candidate compounds
20 positively identified from a chemical or natural product library may be significantly enhanced if the library is screened against both the L- and D-version of the enzyme. For example, screening a natural product library with respect to the inhibition of the
25 protease activity of both L- and D-HIV protease should significantly increase the number candidate drugs identified as active inhibitors.

The above concepts apply equally to screening natural product libraries with respect to receptor activity, i.e. as agonist or antagonist with respect to protein receptors. Included amongst protein receptors against which natural product and/or chemical libraries may be usefully screened are the following: GPIIb-IIIa and LFA-1, Ruoslahti et al.,
30 Science, 238: 491-497 (1987); CSAT, Horwitz et al., J. Cell Biol., 101:2134 (1985); VLA-2, Nieuwenhuis et al. Nature, 318:470 (1985); CR3 Complement Receptor,
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Wright et al., *PNAS*, 84:1965 (1987); CR2 Complement Receptor, Nemerow et al., *J. Virol.*, 55: 3476 (1985); CD4 T Cell Receptor, Guyader et al., *Nature*, 320:662 (1987); FRP Receptor, Yu et al., *Nature*, 330:765 (1987); Apolipoprotein Receptor, Yamada et al., *J. Clin. Invest.*, 80: 507 (1987); Interleukin Receptor, Dower et al., *Immunology Today*, 8:46 (1987); Fc Receptor, Anderson et al., *J. Immunol.*, 138: 2254 (1987); Somatostatin Receptor, Kim et al., *J. Biol. Chem.*, 262: 470 (1987); PDGF Receptor, Keating et al., *J. Biol. Chem.*, 252: 7932 (1987); and Transferrin Receptor, Kohgo et al., *Blood*, 70:1955 (1987).

Other proteins having binding sites which may be screened according to the method of the present invention include insulin receptor binding site on insulin, reovirus receptor binding site on the firal hemaglutinin protein, fibrinogen receptor binding site on figrinogen A alpha, thyroid hormone receptor binding sites α and β , LDL receptor binding site on Apo E, lipid A binding site, lecithin-cholesterol acyltransferase (LCAT) binding site on Apo AI, and Mac-1 integrin receptor binding site on fibrinogen D-30.

E. Methods for Producing Chirally Pure Compounds
In another embodiment, the present invention contemplates methods using a D-enzyme of this invention for the production of chirally pure chemical compounds. A chirally pure compound, as used herein refers to a molecule substantially free from its stereoisomer.

The methods can be practiced in a variety of ways. A single chirally pure chemical can be produced by a reaction by D-enzyme upon a racemic mixture of substrates, leaving in the racemic mixture one of the substrate isomers, and converting the other substrate isomer into a product. In this method, the desired

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chirally pure compound can be a reaction product, or it can be the substrate isomer left unreacted in the racemic mixture, freed from the contaminating isomer by the action of the D-enzyme.

5 Thus, in this embodiment, the invention contemplates a method of producing a chirally pure chemical comprising:

10 a) reacting in an aqueous admixture a first stereoisomer with a D-enzyme that specifically converts said first stereoisomer into a chiral reaction product; and

15 b) isolating the chiral reaction product from the admixture, thereby forming the chirally pure chemical. In an alternative version of this embodiment, the aqueous admixture comprises a racemic mixture having at least a first and a second stereoisomer.

20 The reaction is initiated by admixing D-enzyme with substrate and subjecting the reaction admixture to suitable reaction conditions for driving the enzyme catalyzed reaction. For a D-enzyme those conditions depend upon the particular reaction chemistry to be catalyzed and upon the conditions under which the enzyme is active. The reaction 25 conditions for a D-enzyme are preferably the same as is optimally used for the corresponding reaction of the isomeric substrate by the corresponding L-enzyme. Preferred reaction conditions are those temperature and aqueous buffer conditions which favor maximum 30 enzyme activity for the desired reaction and minimum undesirable side reactions.

The isolating step can be conducted by any chemical manipulation that provides for the resolution of the chirally pure chemical from the 35 reaction product admixture formed in step (a). Exemplary isolating manipulations are well known to the chemist and include solvent extractions,

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chromatography, selective crystallization, distillation, and the like.

In a related embodiment, the invention contemplates a method for producing a chirally pure 5 chemical comprising:

a) reacting in an aqueous admixture a racemic mixture having at least a first and a second stereoisomer with a D-enzyme that specifically converts the first stereoisomer into a reaction 10 product; and

b) isolating said second stereoisomer from said admixture, thereby forming said chirally pure chemical. The reaction is conducted for a time period and under reaction conditions sufficient to 15 convert substantially all of the first stereoisomer into the chiral reaction product.

In this latter embodiment, depletion of the original racemic mixture of an undesirable stereoisomer resolves the chirally pure chemical, and 20 the remaining unreacted stereoisomer is isolated to form the chirally pure chemical.

Chemical synthesis of a chirally pure chemical using a D-enzyme can be conducted in a homogeneous or heterogeneous aqueous reaction environment, or can be 25 conducted in enzyme reactors, where the D-enzyme is in the solid phase, or in membrane reactors, where a solvent or D-enzyme is segregated away from either the reactants or products. Such solid phase enzyme reactors and membrane enzyme reactors, and their methods of use, have been extensively described in 30 United States Patent Nos. 5,077,217, 5,057,427, 4,800,162, and 4,795,704, which are specifically incorporated herein by reference.

35 F. Methods of Co-Crystallization Racemic Mixtures

In one embodiment, the invention contemplates the use of a D-enzyme to produce an X-diffraction pattern of a crystal for determining the three dimensional structure of a protein. Methods for preparing crystallized proteins and analyzing the crystal structure by the X-ray crystallographic arts is well known. See for example the teaching of Stout et al., in "X-Ray Structure Determination: A Practical Guide", Macmillan, New York, 1968; and Miller et al, 10 J. Mol. Biol., 204:211-212, 1988, which are hereby incorporated by reference.

In a preferred embodiment, the invention contemplates the co-crystallization of the D- and L-HIV-1 protease enzymes prepared as described in Example 1. The resulting crystal, formed by the vapor diffusion crystal growth method described by Miller et al., supra, is used to solve the three dimensional structure of HIV-1 protease using conventional X-ray diffraction methods to produce a 15 racemic crystal due to the presence of the enantiomeric forms (D- and L-) of HIV-1 protease in the crystal.

Such a crystal structure is further useful, for example, to model inhibitors of HIV-1 protease useful 25 for therapeutic treatment of HIV-1 infection by inhibition of the protease.

Co-crystallization of D- & L- HIV protease preparations can generate centrosymmetric crystals for high-accuracy X-ray diffraction studies. This 30 should provide data that can find a wide usefulness in drug design studies for AIDS therapeutics. For this purpose, it is necessary to produce each enantiomorph of the enzyme in hundred milligram quantities without the complication of autolysis 35 during the extraction, purification and folding of the synthetic products. We have disclosed that an HIV-1 protease analogue, prepared by the directed

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chemical ligation of unprotected peptide segments and containing a thioester replacement for the natural peptide bond between Gly⁵¹-Gly⁵², has full activity. This segment condensation strategy also largely 5 avoids the possibility of autodigestion by the enzymes during their preparation.

G. Therapeutic Methods

The invention contemplates therapeutic methods involving administration of therapeutically effective 10 amounts of a D-enzyme to a mammal or human, where an L-enzyme would normally be the active ingredient in the therapeutic composition to be administered. By substituting a D-enzyme for its corresponding L-enzyme, the therapeutic enzyme acquires the benefits 15 of a D-enzyme as described herein, including increased effective half-life due to resistance to proteases, and diminished immune recognition.

Enzymes for use in therapeutic treatment methods as a D-enzyme of this invention can be derived from 20 any number of enzymes of known primary amino acid residue sequence that provide therapeutic applications, and which have achiral substrates. Antioxidant enzymes, in particular, have therapeutic applications that can benefit from being in the form 25 of a D-enzyme to increase effective therapeutic half-life.

Antioxidants function as anti-inflammatory agents. The medically important antioxidant enzymes of known structures are superoxide dismutase, catalase and 30 glutathione peroxidase. These enzymes are involved in the prevention of post-ischemic injuries and the control of inflammatory disorders. Wilsman et al., In: Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine, Rotilio, Ed., Elsevier Science, Amsterdam Publishers (1986). As 35 demonstrated in the case of SOD, these enzymes would benefit from an increased circulatory half-life.

1. Superoxide Dismutase

The SOD enzymes, which catalyze the conversion of superoxide radical to molecular oxygen and hydrogen peroxide, are ubiquitous in organisms
5 that utilize oxygen.

The dismutation reaction of SOD enzymes is important in preventing tissue damage by free radicals. Indeed, the effectiveness of human intracellular SOD (HSOD) in relieving inflammatory disorders including osteoarthritis has been demonstrated by clinical studies in humans. See, Wilsmann, Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine, Elsevier, 500-5 (1986). Additionally, animal studies have suggested
10 that SOD enzymes have therapeutic potential for viral infections. See, Oda et al., Science, 244:974-6 (1989). SOD enzymes have also been implicated in preventing alloxan diabetes [Grankvist et al.,
15 Nature, 294:158 (1981)] and in preventing metastasis of certain forms of cancer (EPO Application No.
20 0332464).

Therefore in one embodiment, methods for reducing tissue damage caused by oxygen free radical (superoxide) in vivo or in vitro are contemplated by
25 the present invention, using a D-superoxide dismutase (D-SOD) enzyme of this invention.

Human recombinant SOD can protect ischemic tissue in experimental models when injected into the circulation just prior to reperfusion (Ambrosio et al. Circulation 75:282. 1987). Injury to the endothelium, a tissue covered with glycosaminoglycans, is a major consequence of ischemia/reperfusion injury. This causes edema formation due to the loss of barrier function and favors platelet adhesion to endothelium. The protective action of SOD is due to its scavenging of superoxide anion. SOD can also protect the

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endothelium "in vivo" by preventing the formation of peroxynitrite, which is toxic due to its decomposition to form potent, cytotoxic oxidants (Beckman et al. Proc. Natl. Acad. Sci. USA 87:1620-1624. 1990). Postischemic injury involving the superoxide anion has been observed in the heart, intestine, liver, pancreas, skin, skeletal muscle, kidney and perhaps occurs in other organs (McCord Fed. Proc. 46:2402-2406. 1987). SOD chemically linked or conjugated to albumin exhibits an increased "in vivo" half-life, i.e. slower clearance. Such conjugated SOD has been shown to be superior to unconjugated SOD with respect to inhibiting postischemic reperfusion arrhythmias (Watanabe et al. Biochem. Pharmacol. 38:3477-3483. 1989). The preparation of D-SOD having a half-life greater than the half-life of L-SOD facilitates the use of SOD for preventing or diminishing postischemic damage.

SOD has also proven to be effective in several inflammatory diseases like osteoarthritis and rheumatoid arthritis. Local infiltration of SOD in extra-articular inflammatory processes (e.g., tendinitis, tendovaginitis, bursitis, epicondylitis, periarthritis) has also proven to be effective.

Improvement upon SOD administration has also been observed in Peyronie's disease and Dupuytren's contracture (Wilsmann. In Rotilio Ed. Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine. Elsevier. 1986). For these inflammatory disorders as well as for respiratory distress syndromes a cell surface targeted SOD with increased half-life will be a useful drug. Organ transport and organ transplant also can benefit from such an improved SOD. In addition, tissue targeted SOD should help alleviate the toxic secondary effect of anti-cancer radio- and chemotherapy. Drug (antibiotic and anticancer) induced nephritis also

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can be reduced by a more potent SOD. D-SOD may be substituted to advantage for L-SOD in the above-recited therapeutic applications.

Thus, the present invention contemplates a method 5 of in vivo scavenging superoxide radicals in a mammal that comprises administering a therapeutically effective amount of a physiologically tolerable composition containing a D-SOD enzyme to a mammal in a predetermined amount calculated to achieve the 10 desired effect.

For instance, when used as an agent for scavenging superoxide radicals, such as in a human patient displaying the symptoms of inflammation induced tissue damage such as during an autoimmune disease, 15 osteoarthritis and the like, or during a reperfusion procedure to reintroduce blood or plasma into ischemic tissue such as during or after surgical procedures, trauma, in thrombi, or in transplant organs, or after episodes of infection causing massive cell death and release of oxidants, the D-SOD 20 enzyme is administered in an amount sufficient to deliver 1 to 50 milligrams (mg), preferably about 5 to 20 mg, per human adult, when the D-SOD enzyme has a specific activity of about 3000 U per mg. A preferred dosage can alternatively be stated as an 25 amount sufficient to achieve a plasma concentration of from about 0.1 ug/ml to about 100 ug/ml, preferably from about 1.0 ug/ml to about 50 ug/ml, more preferably at least about 2 ug/ml and usually 5 to 10 ug/ml.

D-SOD enzymes having superoxide dismutase (SOD) activity for use in a therapeutic composition typically have about 200 to 5000 units (U) of enzyme activity per mg of protein. Enzyme assays for SOD 35 activity are well known, and a preferred assay to standardize the SOD activity in a D-enzyme is that

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described by McCord et al., J.Biol.Chem., 244:6049 (1969).

For treating arthritic conditions such as rheumatoid arthritis, tendinitis, bursitis or the like, a dosage of about 1 to 20 mg, preferably about 4 to 8 mg is administered intra articulately per week per human adult. In certain cases, as much as 20 mg can be administered per kilogram (kg) of patient body weight.

For treating reperfusions, or myocardial injuries, a dosage of 5 mg per kg of body weight is preferred to be administered intravenously.

The therapeutic compositions containing a D-SOD enzyme are conventionally administered intravenously, or intra articulately (ia) in the case of arthritis, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for initial administration and booster shots are also

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variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous 5 intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

Additional exemplary therapeutic applications of SOD, which are directly applicable to the present 10 methods of using therapeutic D-SOD, and compositions containing SOD useful therefor, are described in United States Patent Nos. 5,084,390, 5,006,333 and 4,656,034, which disclosures are specifically incorporated herein by reference.

15 Similarly to the improved therapeutic methods described above with D-SOD, many otherwise satisfactory enzyme pharmaceutical agents are expected to find limited therapeutic use due to their short lifetimes in vivo. Thus, a convenient method 20 for extending the useful lifetimes of proposed pharmaceutical agents is desired and is provided by the preparation of a D-enzyme according to the present invention. The methods herein allow the preparation of the D-enzyme variants of the enzyme 25 pharmaceutical agents that at least have biological activities comparable to those for the unaltered agent.

H. Therapeutic Compositions

30 Many of the compounds and groups involved in the instant specification (e.g., D-amino acid residues) have a number of forms, particularly variably protonated forms, in equilibrium with each other. As the skilled practitioner will understand, 35 representation herein of one form of a compound or group is intended to include all forms thereof that are in equilibrium with each other.

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In the present specification, "uM" means micromolar, "ul" means microliter, and "ug" means microgram.

5 Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with a D-enzyme, as described herein, dissolved or dispersed therein as an active ingredient.

10 As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological 15 effects such as nausea, dizziness, gastric upset and the like.

20 The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be 25 emulsified.

30 The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active 35 ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable

salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

A therapeutic composition contains an amount of a D-enzyme of the present invention sufficient to deliver a catalytic amount of the enzyme to the target tissue to be treated. Typically this is an amount of at least 0.1 weight percent, and more preferably is at least 1 weight percent, of D-enzyme per weight of total therapeutic composition. A weight percent is a ratio by weight of protein to total composition. Thus, for example, 0.1 weight

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percent is 0.1 grams of D-enzyme per 100 grams of total composition.

Examples

5 The following examples are intended to illustrate, but not limit, the scope of the invention.

1. Conventional Step-wise Synthesis of L- and D-[Aba^{67,95,167,195}]HIV-1 Protease (HIV-1 PR)

10 Advances in the total chemical synthesis of proteins made possible the reproducible production of homogeneous crystalline L-[Aba^{67,95,167,195}]HIV-1 protease (HIV-1 PR). See, for example Kent, Annu. Rev. Biochem., 57:957, (1988); Wlodawer et al., Science, 245:616 (1989); and Miller et al., Science, 246:1149 (1989).

20 HIV-1 protease (HIV-1 PR) is a virally-encoded enzyme which cuts polypeptide chains with high specificity and which is essential for the replication of active virions. Kohl et al., Proc. Natl. Acad. Sci., U.S.A., 85:4686, 1988. The 21,500 dalton HIV-PR molecule is made up of two identical 99 amino acid polypeptide chains.

25 The total chemical synthesis of D-[Aba^{67,95,167,195}]HIV-1 protease was carried out as described below, and the properties (covalent structure, physical properties, circular dichroism, enzymatic activity) of the D- and L- enantiomeric forms of this HIV-1 protease enzyme were compared.

30 To that end, in separate chemical syntheses, the protected polypeptide chains corresponding to the L- and the D-sequences of the [Aba^{67,95}] HIV-1 protease 99-aa monomer were prepared by total chemical synthesis. Aba is L- or D-a-amino-n-butyric acid and is used as an isosteric replacement for Cys residues at positions 67 and 95 in the HIV PR monomer polypeptide chain. This same isosteric replacement

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was used in the work of Wlodawer et al., supra, and Miller et al., supra, leading to the original correct structures of HIV PR.

The chemical synthesis was conducted in conventional stepwise fashion. The 99-aa polypeptide chains were assembled from protected L-amino acids and protected D-amino acids, respectively. The t-Boc D- and L-amino acid derivatives were obtained from the Peptide Institute (Osaka, Japan) and Peptides International (Louisville, KY) except: Boc-L-Aba, Boc-L-Asn(Xan), Boc-D-Ile and Boc-D-His(Bom), obtained from Bachem Bioscience (Philadelphia, PA); Boc-D-Asn(Xan), Boc-D-Asp(OcHex) and Boc-D-Glu(OcHex), obtained from Bachem California, (Torrance, CA); Boc-D-Lys(ClZ), crystallized from the TBA salt obtained from the Peptide Institute; and, D-Aba (Sigma, St. Louis, MO) which was converted to Boc-D-Aba and isolated as the DCHA salt. Other side chain protecting groups that were used were: Arg(Tos), Tyr(BrZ), L-His(Tos), D-His(Bom) and Thr(BzL). The L-enantiomer content of the Boc-D-amino acid preparations was between 0.01 and 0.08% [manufacturers specifications]. Stepwise chain assembly was carried out in machine assisted fashion on an Applied Biosystems 430A synthesizer (0.2 mmole scale with D- or L-Boc-Phe-OCH₂-Pam-resin). Each cycle of amino acid addition involved: N^α-deprotection, neat (100%) TFA [2x30 sec flow washes, plus 1 minute batchwise treatment]; DMF flow wash [1x22 sec, 1x38 sec]; coupling [1x10 minute] with simultaneous in situ neutralization [Boc-amino acid (2.25 mmol) preactivated by reaction with HBTU (2.22 mmol) and DIEA (6.4 mmol) in DMF for 2 min]. The in situ neutralization method has been shown to result in negligible levels of racemization. Henklein et al., in "Innovation & Perspectives in Solid Phase Synthesis", R. Epton Ed., SPPC Ltd., Birmingham,

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U.K., 1992. The assembled peptides were deprotected and cleaved from the resin in 9:1 HF/p-cresol (resorcinol and thiocresol were present when His(Bom) was included in the sequence) after removal of the 5 Boc group and the formyl group from Trp (with ethanolamine).

The D- and L- products after deprotection were worked up individually, and synthetic enzymes were then prepared by folding the polypeptide polymers 10 from denaturant as described by Wlodawer et al., supra, and Miller et al., supra. To that end, after deprotection and cleavage, the crude peptide products were precipitated with ether and dissolved with 6M guanidine hydrochloride in a pH 8.0 NaHCO₃ buffer 15 prior to semi-preparative C18 RP HPLC enrichment and folding by dialysis in 10% glycerol, 25mM NaH₂PO₄ buffer pH 7.0. After concentration under high vacuum to a solution in glycerol, the enzymes were quantitated by amino acid analysis and stored at 4° C.

20 Total yield for the synthesis of L-[Aba^{67,95,167,195}] HIV-1 Protease (HIV-1 PR) was approximately 2 milligrams or 0.09%.

25 Total yield for the synthesis of D-[Aba^{67,95,167,195}] HIV-1 Protease (HIV-1 PR) was indeterminant because it was less than 2 milligrams.

2. Structural Analysis of the above L- and D-[Aba^{67,95,167,195}] HIV-1 Protease (HIV-1 PR)

30 The D-enzyme 99-aa monomer, D-[Aba^{67,95}] HIV-1 protease, was analyzed for various structural characteristics, and compared to the structural characteristics of the L-isomer. For example, analytical reversed-phase HPLC gave identical retention times for the two synthetic polypeptide 35 chains.

Purified, folded chemically synthesized [Aba^{67,95}]HIV-1 protease monomer samples prepared in

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Example 1 in pH 6.5 MES buffer/10%glycerol were subjected to desalting by reverse phase HPLC. The collected protein peaks were each separately analyzed by ion spray mass spectrometry as described by Bruins et al., Anal. Chem., 59:2642 (1987). Under the conditions used (50% acetonitrile, 50% water, 0.1%TFA) the enzyme is denatured. In the reconstructed mass spectra shown, the raw m/z data have been subjected to a high pass digital filter, then sorted to yield all parent molecular species between 10kDa and 11kDa. This reconstruction procedure mathematically reduces the multiple charge states observed for a given molecular species to a single molecular mass.

By reconstructed ion spray mass spectroscopy, the observed monomer molecular mass of the L-enzyme was 10,748 \pm 4 daltons (Da), and the mass of the D-enzyme was 10,751 \pm 3 Da. Calculated mass: (monoisotopic) 10,748.0 Da; (average) 10,754.7 Da.

Thus, the two products (D- and L- enzyme monomers) had the same molecular weight, within experimental uncertainty, when measured by ion spray mass spectrometry. The ion spray mass spectrometry data are shown in Figure 1.

The complete amino acid sequence of the D-enzyme 99-aa monomer was determined by matrix-assisted laser desorption time-of-flight mass spectrometric readout (Model API-III mass spectrometer, P.E. SCIEX Inc., Thornhill, Toronto, Canada) and was shown to be the same as that of the L-enzyme. Thus, the two synthetic enzyme molecules had identical covalent structure.

On the other hand, differences between the two molecules were revealed in various chiral interactions. Circular dichroism (CD) spectra of the individual D- and L-HIV-1 protease enantiomers were taken over the range 260-195 nm in a pH 5.5 aqueous solution containing 5% glycerol at 25°C using a 1 mm

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path length quartz cell on an Aviv CD spectrometer. The CD spectra revealed equal and opposite optical rotations, as expected for enantiomeric protein molecules.

5

3. Enzymatic Properties of the above L- and D-
[Aba^{67,95,167,195}]HIV-1 Protease (HIV-1 PR)

The enzymatic properties of the enantiomeric protein comprised of D-amino acids was evaluated and compared to the L-isomer using a fluorogenic assay which employed a hexapeptide analog of a natural GAG cleavage site as substrate as described by Toth et al., Int. J. Peptide Protein Res., 36:544 (1990).

The fluorogenic assays were performed with 15 ul aliquots (corresponding to 1.75 ($\pm 10\%$) ug protein) of each enzyme enantiomer in 10% glycerol, 100mM MES buffer pH 6.5 added to a solution of 50 mM D- or L-fluorogenic substrate in the MES buffer. The substrate for the enzyme had the sequence
20 2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO₂)-Gln-Arg.amide.
(SEQ ID NO 2). The substrate was synthesized with either D- or L-amino acid derivatives to provide the appropriate enantiomeric forms.

The results of the fluorogenic assay are shown in Figure 2. Aliquots containing equal amounts (as determined by amino acid analysis) of the purified, folded enzyme preparations were used in the fluorogenic assay. The increase in fluorescence was recorded on a continuous chart recorder. The data illustrate that the two synthetic enzyme molecules were equally active, but revealed a reciprocal chiral specificity in that the L-enzyme cleaved only the L-substrate while the D-enzyme cleaved only the corresponding D-substrate.

35 In a similar study, the D- and L- enantiomers of the pseudopeptide inhibitor, MVT101 (Ac-Thr-Ile-Nle-psi[CH₂NH]-Nle-Gln-Arg.amide), (SEQ ID

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NO 1) prepared as described by Miller et al., Science, 246:1149 (1989), were evaluated for their effect on the D- and L-HIV protease. The results of the studies using inhibitor are shown in Table 1.

5

TABLE 1
Chiral inhibitors show reciprocal chiral specificity
against D- and L-HIV PR^a.

	L-MVT101	D-MVT101	Evans Blue ^b
--	----------	----------	-------------------------

L-HIV PR	+	-	+
D-HIV PR	-	+	+

20 "The D- and L-enzymes were separately assayed by the fluorogenic assay method described above using the corresponding chiral substrate, in the presence of 5xIC50 concentration of inhibitor.

25 ^bThe inhibitor Evans Blue is a non-peptide, achiral mixed competitive-uncompetitive inhibitor of the HIV-1 PR.

30 The chiral inhibitors were effective only against the corresponding enantiomer of the enzyme, i.e. L-MVT101 inhibited L-HIV PR but not the D-HIV PR-catalyzed reaction, and D-MVT101 inhibited D-HIV PR but had no effect on the L-enzyme-catalyzed reaction. Interestingly, the achiral inhibitor Evans Blue, which shows mixed inhibition kinetics, was a potent inhibitor of both enantiomers of the enzyme (Table 1).

35 The HIV-1 protease exists as a homodimer; that is, a single enzyme molecule is made up of two identical

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99 residue folded polypeptide chains. Wlodawer et al., Science, 245:616 (1989); and Miller et al., Science, 246:1149 (1989). HIV PR is highly active, showing rate enhancement of about 10¹⁰-fold over uncatalyzed peptide-bond hydrolysis. Kent et al., in "Viral Proteinases as Therapeutic Targets", Wimmer et al., Eds., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989, pp. 223-230; and Richards et al., FEBS Lett., 247:113 (1989). It is a highly specific enzyme which cleaves peptides as well as proteins (Kent et al., supra; and Kröusslich, et al., Proc. Natl. Acad. Sci. USA, 86:807, 1989) and its specificity is determined by the interactions of the three dimensionally folded enzyme molecule forming a complex with six consecutive amino acid residues in the substrate polypeptide chain. Miller et al., Science, 246:1149 (1989); and Kent et al., supra.

As with all enzymes, HIV PR owes its specificity and catalytic activity to the precise three dimensional structure formed by specific folding of the polypeptide chain, and to precise geometric interactions in the specific complexes formed with substrates. Fersht, in "Enzyme Structure and Mechanism", W.H. Freeman and Company, San Francisco, 1977, pp. 75-81. The observed reciprocal chiral specificities therefore show that the folded forms of the D- and L-enzyme molecules are mirror images of one another in all elements of the three dimensional structure responsible for the enzymatic activity. The extensive nature of these interactions implies that the two enzyme molecules are mirror images in every respect (21), consistent with the observed equal and opposite CD spectra. Most notably, the folded form of the polypeptide backbone (i.e. ignoring the side chains) is itself a chiral entity that must exist in mirror image form in the two protein enantiomers as shown in Figure 3.

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The ribbon representation of L- and D-[Aba67,95,167,195]HIV-1 Proteases shown in Figure 3 is based on the X-ray crystallographic coordinates of the chemically synthesized enzyme when complexed with 5 a substrate-derived peptide inhibitor (inhibitor is not shown) as described by Miller et al., Science, 246:1149 (1989). This model was generated by performing a mirror image transformation of the L-enzyme data.

10 The folded three-dimensional ribbon "backbone" structures are non-superimposable mirror images and contain numerous chiral elements. These are found in secondary and supersecondary structure, in the tertiary structure and in the quaternary structure as 15 illustrated in Figure 3. Note, for example, the relatedness of the flaps to one another; the relatedness of the helix segments to the neighboring β -strands; the characteristic twist (right-handed, in the L-protease) of the antiparallel β -strands in each 20 flap described by Richardson et al., in "Protein Folding", Gierasch et al., Eds., American Association of the Advancement of Science, Washington, D.C., 1990, pp.5-17.; and, the handedness of the helical segments. Since the only chiral element introduced 25 in the chemically synthesized polypeptide chains is the stereochemistry at the amino acid C α atoms (and the C β atoms of Thr, Ile), the data presented herein demand that all stereochemical aspects of the folded enzyme molecule, from secondary to quaternary 30 structure, are determined simply by the stereochemistry of the polypeptide backbone. Thus, the present reciprocal chiral properties of the chemically synthesized enzyme enantiomers is a fundamental demonstration that the final folded/three 35 dimensional structure and consequent biological activities of this 21500 dalton homodimeric enzyme

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molecule are completely determined by the amino acid sequence.

The L- and D-enzymes in this study have never seen biosynthetic conditions, and have thus never been in contact with biochemical factors of any sort.

Interestingly, the simple homodimeric enzyme molecule studied here is formed rapidly (both folding and assembly) and accurately even at the relatively low concentrations used in the assay conditions, as well as in more normal dialysis-from-denaturant folding conditions. The results described herein are conclusive evidence that whatever their proposed role, biosynthetic factors are not required for the formation of the correct, functional folded and assembled form of the protein.

The observed reciprocal chiral properties of the mirror-image enzyme molecules described herein reinforces and generalizes the chiral nature of biochemical interactions of proteins. The chiral properties of the protein molecules themselves, which give rise to this behavior, are given only cursory attention in biochemical texts. We can now state, based on experimental evidence, that protein enantiomers will display reciprocal chiral specificity in their biochemical interactions including catalysis.

The observation that both enantiomers of HIV PR were equally affected by the achiral inhibitor Evans Blue provides a number of significant implications. First, the unnatural enantiomer of an enzyme that operates on an achiral substrate and yields an achiral product will be fully functional *in vivo*. This aspect provides important potential therapeutic applications. Examples are carbonic anhydrase and superoxide dismutase. D-Enzymes are expected to be long lived *in vivo* (in an L-protein biosphere), since they will be resistant to naturally occurring

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proteases which will in general attack only proteins made up of L-amino acids. D-proteins are comparatively less immunogenic than L-proteins because long polypeptides made up entirely of D-amino acids are not processed and presented as efficiently by the immune system as are polypeptides made up of L-amino acids.

D-Protein molecules have other potential practical applications. For example enzyme enantiomers have utility as chiral catalysts in the selective production of a pure enantiomer of a fine chemical. Enantiomerically pure chemical synthesis has applications to the production of human pharmaceuticals. In addition, protein enantiomers can contribute to the acquisition of phase data in X-ray crystallography as described by Mackay, Nature, 342:133 (1989). Centro-symmetric crystals formed by the co-crystallization of a D-, L-protein pair would have greatly simplified phases, and provide more reliable X-ray structural data. At the present time D-enzymes, and D-proteins in general, are accessible only by total chemical synthesis. During ribosomal synthesis of polypeptide chains, even in vitro translation systems, D-amino acids will not be incorporated into growing polypeptides. Ellman et al., Science, 255:197 (1992).

4. Discussion of Examples 1-3

D- and L- forms of the enzyme HIV-1 protease are prepared herein by total chemical synthesis. The two proteins have identical covalent structures. However, the folded protein/enzyme enantiomers show reciprocal chiral specificity on peptide substrates. That is, each enzyme enantiomer cuts only the corresponding substrate enantiomer. Reciprocal chiral specificity was also evident in the effect of the enantiomeric inhibitors of the HIV-1 protease enzymes prepared herein. These data show that the folded forms of the

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chemically synthesized D- and L-enzyme molecules are mirror images of one another in all elements of the three dimensional structure. Enantiomeric proteins display reciprocal chiral specificity in all aspects 5 of their biochemical interactions, retain enzymatic activity, and provide a wide range of useful compositions as described herein.

5. Synthesis and Ligation of D- and L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂ HIV-1 Protease Analogs.

Figure 4 illustrates a schematic representation of the strategy employed for the total synthesis of the D- and L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂ HIV-1 protease analogues. Protected D- and L-amino acids may be obtained from 10 the Peptide Institute (Osaka, Japan), Peptides International (Louisville, KY), Bachem Bioscience (Philadelphia, PA) and Bachem California (Torrance, CA) and had <0.03% of the opposite enantiomer. HPLC purified, functionalized, unprotected peptide 15 segments, assembled by stepwise solid-phase synthesis, is reacted in the presence of 6M GuHCl to form the ligated 99-residue D- and L-[(NHCH₂COSCH₂, CO)⁵¹⁻⁵²] HIV-1 PR products. (Schnölzer et al., (1992) Science 256, 221-225.) The boxed area of Figure 4 20 represents the structure of the thioester analogue of the peptide bond Gly⁵¹-Gly⁵² at the site where the ligation occurred. The thioester serves as a link between the two D-peptides, i.e. the site of 25 ligation. A selenol ester linkage may also be employed for ligating two D-peptides.

Two large peptide segments, i.e. [α COSH]HIV-1 PR(1-51) and [N^α-BrCH₂CO]HIV-1 PR(53-99), are 30 assembled, as illustrated in Figure 5, in separate syntheses by a highly optimized machine-assisted SPPS protocol using Boc-chemistry performed on a modified ABI 430A synthesizer. (Kent et al., "Innovation & Perspectives in Solid-Phase Synthesis", (1992) Ed.

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Epton, R. SPPC Ltd. Birmingham, U.K.). The protocol comprised removal of the Na-Boc group with undiluted TFA (2 minute total) followed by a DMF flow wash to give the TFA-peptide-resin salt, and a single 10 minute coupling step using HBTU activated Boc-amino acids and in situ neutralization with DIEA in DMF. Deprotection and coupling reactions are separated by a flow wash step. After purification, the monomers are separately folded by dialysis in the presence of D- & L- MVT-101 inhibitor, respectively, to yield the homodimeric enzymes. The milligram yields of each product are provided in Figure 4.

The [α COSH]HIV-1 PR(1-51) peptide may be assembled on 4-[a(Boc-Gly-S)benzylI]phenoxyacetamidomethyl-resin. The [N^{α} -BrCH₂CO]HIV-1 PR[53-99] peptide may be prepared by bromo-acetylation of [Aba^{67,95}]HIV-1 PR(53-99)-OCH₂ Pam peptide-resin. (Yamashiro et al., (1988) *Int. J. Peptide Protein Res.* 31, 322-334.) All peptides are cleaved and deprotected by high HF treatment.

After preparative reverse phase HPLC purification (Vydac 218TP101550 - 5x25cm, 0.1% TFA/CH₃CN & 30-50ml/min) the functionalized peptide segments are reacted in the presence of 6M GuHCl (in 0.1M phosphate buffer, pH 5.3) for 48 hours to form the ligated D-and L-[$(NHCH^2COSCH_2CO)^{51-52}$]HIV-1 PR monomers. Figure 6 illustrates a composite chromatogram showing the two purified functionalized unprotected segments and the final (48 hour) ligation reaction product (bold) run on a Vydac 218TP5415 column eluted with 0.1% TFA (buffer A) and 0.9% TFA/CH₃CN, 1:9 (buffer B), at a flow rate of 1 ml/min.

After purification by reverse phase HPLC, the products may be separately folded by dialysis in 25 millimolar phosphate buffer, pH 5.5, in the presence of a 10-fold excess of either D- or L-[MVT-101] inhibitor (Ac-Thr-Ile-Nle- ψ -[CH₂NH]-Nle-Gln-Arg.NH₂)

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(SEQ ID NO 1) to yield the homodimeric enzymes.
(Miller et al., (1989) *Science* **246**, 1149.) Step
reaction yields for the synthesis of the D- and L-
[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂HIV-1 protease analogs are provided
5 by Figure 7.

Total yield with respect to the synthesis of the
D-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂, HIV-1 protease analog was 48
milligrams or 3.0%.

10 Total yield with respect to the synthesis of the
L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂, HIV-1 protease analog was 47
milligrams or 2.5%.

15

6. Physical Characterization of the Ligation
Products, D- and L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂HIV-1
Protease Analogs

Ion spray mass spectrometry of the HPLC purified
ligated products is illustrated in Figure 8. Ion
spray mass spectrometry reveals single molecular
species in each case with observed molecular masses
20 of 10768.9±1.4 daltons (D-enantiomer) and 10769.4±0.9
daltons (L-enantiomer) [Calculated: 10763.9 daltons
(monoisotopic) and 10770.8 daltons (average)]. Minor
amounts of dehydration byproducts were also detected.
The sequences of the monomers were also examined by a
25 new protein ladder sequencing technique utilizing a
one step laser desorption mass spectrometric readout.
Figure 8 (A & C) illustrate labelled peaks
representing a single molecular species differing in
the number of excess protons. The observed molecular
masses of the ligated products is 10768.9±1.4 daltons
30 (D-enantiomer) and 10769.4±0.9 daltons (L-enantiomer)
[Calculated: 10763.9 daltons (monoisotopic) and
10770.8 daltons (average)]. Figure 8 (B & D)
illustrate reconstructed mass spectra in which the
35 raw data shown in A & C has been reduced to a single
charge state. All data points in A & C are included
in the calculations and no mathematical filtering is

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performed. The mass regions from 10 to 11 kD are shown for clarity.

Figure 9 illustrates the reverse phase HPLC measurements of D- & L-[Aba^{67,95},(CO-S)⁵¹⁻⁵²] HIV-1 PR ligation products. The ligated products from 6M GuHCl reveal a single peak in each case. Panels A and C illustrate the purified ligated monomers in 6M GuHCl. Panels B and D illustrate the homodimeric enzymes folded in the presence of D- or L-[MVT-101] inhibitor, respectively, in 25 millimolar sodium phosphate buffer, pH 5.5. Note that, after folding, a number of minor autolysis products are seen in both the D- and L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂HIV-1 protease preparations. At approximately 27 minutes, minor proteolytic products of the MVT-101 inhibitor peptide are also seen. It would seem that even in the presence of a large excess of inhibitor, the enzyme is still subject to a minor degree of autolysis. The samples were run on a Vydac 218TP5415 column eluted with 0.1% TFA (buffer A) and 0.9% TFA/CH₃CN, 1:9 (buffer B), at flow rate of 1 ml/min.

Figure 10 illustrates the far-ultraviolet circular dichroism spectra of the folded D- and L-protease preparations. The spectra were recorded in 25 millimolar sodium phosphate buffer, pH 5.5 (0.4 mg/ml protease in the presence of inhibitor) at 25°C in a quartz cell with a pathlength of 1 millimeter. Each preparation is of equal magnitude, but opposite sign, as expected for mirror image proteins.

(Corigliano-Murphy et al., (1985) *Int. J. Peptide Protein Res.* 25, 225; and Zawadzke et al., (1992) *J. Am. Chem. Soc.* 114, 4002).

7. Characterization of the Enzymatic Activity of
35 Ligation Products, D- and L-[Aba^{67,95}(CO-S)⁵¹⁻⁵²]₂,
HIV-1 Protease Analogs

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Figure 11 illustrates the enzymatic activity of the D- & L-[Aba^{67,95}(CO-S)^{51,52}]₂ HIV-1 PR enantiomers may be determined by their action on D- and L-isomers of the hexapeptide substrate Ac-Thr-Ile-Nle-Nle-Gln-Arg.amide (an analog of the p24/p15 GAG viral processing site). The D-enzyme cleaves only the D-substrate and is inactive on the L-substrate, while the L-enzyme shows full activity towards the L-substrate, but is inactive towards the D-substrate.

This reciprocal chiral specificity is also evident in the effect of chiral inhibitors. As shown in the Table, D-and L-[MVT-101] inhibits the cleavage of chiral fluorogenic substrates by the D- and L-HIV-1 PR analogues respectively, but has no effect on the action of the opposite enantiomer. Interestingly, the achiral inhibitor, Evans Blue, which shows mixed inhibition kinetics, inhibits both enantiomers of the enzyme.

20

Table

Chiral inhibitors show reciprocal chiral specificity against D- and L-[Aba^{67,95}(CO-S)^{51,52}]₂ HIV-1 PR *

		L-MVT101	D-MVT101	Evans Blue
25	D-[Aba ^{67,95} (CO-S) ^{51,52}] ₂ HIV-1 PR	+	-	+
	L-[Aba ^{67,95} (CO-S) ^{51,52}] ₂ HIV-1 PR	-	+	+

30

The D- and L-enzymes were separately assayed by the fluorogenic assay method using the corresponding chiral substrate, in the presence of 5 x IC₅₀ concentration of inhibitor. The fluorogenic assays were performed with 15 ul aliquots (corresponding to 1.75 (\pm 10%) mg protein) of each enzyme enantiomer in 100 millimolar MES buffer pH 6.5 added to a solution

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of 50mM D- or L-fluorogenic substrate in the MES buffer. The substrate sequence was 2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO₂)-Gln-Arg amide (SEQ ID NO 2): it was synthesized with either D- or L-amino acid derivatives to provide the appropriate enantiomeric forms. The inhibitor Evans Blue is a non-peptide, achiral mixed competitive-uncompetitive inhibitor of the HIV-1 PR enzyme.

The enzymatic activity of the [Aba^{67.95}, (CO-S)⁵¹⁻⁵²]₂ HIV-1 PR enantiomers with respect to the D- and L-isomers of the substrate Ac-Thr-Ile-Nle-Nle-Gln-Arg.amide (SEQ ID NO 3) may be measured as follows. The substrate (1mg/ml) is treated with enzyme (0.1mg/ml) at pH6.5 (MES buffer, 100mM) for 30 minutes at 37°C. An aliquot of the reaction mixture is then chromatographed (Vydac 218TP5415 RP HPLC column) with a linear gradient, 0-40%, of buffer B (0.09% TFA/CH₃CN, 1:9) in buffer A (0.1% TFA) over 20 minutes (flow rate 1ml/min, A^{214nm}). The peptide products are identified by ion spray MS as (H)-Nle-Gln-Arg.amide (m/z: 415.0 - early eluting) and Ac-Thr-Ile-Nle-(OH) (m/z: 388.0 - late eluting). Minor impurities present in the substrate preparations due to unpurified peptides are not cleaved. Panel A illustrates D-substrate only; panel B illustrates L-substrate only; panel C illustrates D-substrate plus D-enzyme; panel D illustrates D-substrate plus L-enzyme; panel E illustrates L-substrate and L-enzyme; panel F illustrates L-substrate plus D-enzyme.

8. Discussion of Examples 4-7

The HIV-1 protease enzyme exists as a homodimeric structure. It is a highly specific enzyme and this specificity and its catalytic activity depend on a precise 3-D structure being formed between the folded dimer and six residues of the substrate molecule.

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The observed reciprocal specificities, therefore, show that the folded forms of the D- and L-enzyme molecules are mirror images of each other in all elements of the 3-D structure responsible for their enzymatic activity. This is consistent with their observed CD spectra.

The 3-D structure of a folded enzyme molecule contains numerous chiral elements in secondary and supersecondary structure, in tertiary structure and in quaternary structure, as illustrated in Figure 3. Since the only difference between the synthetic D- and L-polypeptide chains is the stereochemistry of the α -carbon atoms (and the C6 atoms of Ile and Thr) of the amino acids, it is concluded that the stereochemistry of the backbone determines all aspects of higher structure in this protein.

The observations of reciprocal chiral specificity in the enzymatic activity of the D- and L-HIV-1 proteases disclosed herein, serve to generalize and emphasize the chiral nature of the biochemical interactions of proteins. The large amounts of high purity D- and L-enzyme enantiomorphs prepared using the chemical ligation method will allow a thorough experimental evaluation of the use of D-, L-proteins in X-ray crystallography.

The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications can be effected without departing from the true spirit and scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: D-ENZYME COMPOSITIONS AND METHODS OF
THEIR USE

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 07-JUN-1993

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/894,817
- (B) FILING DATE: 08-JUN-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /label= N-acetyl
/note= "An N-acetyl group is located at the amino terminus of the peptide."

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(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /label- Nle
/note- "The modified amino acid, Nle, is present at this position."

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /label- Nle
/note- "The modified amino acid, Nle, is present at this position."

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: (3^4)
- (D) OTHER INFORMATION: /label- psiCH2NH
/note- "The scissile peptide bond between these amino acids has been replaced by a reduced analog, psiCH2NH."

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /label- Amide
/note- "An amide is located at the carboxy terminus of the peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Ile Xaa Xaa Gln Arg
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note- "A 2-aminobenzoyl group is located at the amino terminus of the peptide."

(ix) FEATURE:

- 57 -

(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /label= Nle
/note= "The modified amino acid, Nle, is present at this position."

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /label= Phe-pNO2
/note= "The modified amino acid, Phe-pNO2, is present at this position."

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /label= Amide
/note= "An amide is located at the carboxy terminus of the peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Ile Xaa Xaa Cln Arg
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /label= Ac
/note= "An acetyl, Ac, group is located at the amino terminus of the peptide."

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /label= Nle
/note= "A modified amino acid, Nle, is located at this position."

(ix) FEATURE:

(A) NAME/KEY: Modified-site

- 58 -

(B) LOCATION: 4
(D) OTHER INFORMATION: /label- Nle
/note- "A modified amino acid, Nle, is located at
this position."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /label- Amide
/note- "An amide is located at the carboxy
terminus of the peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ile Xaa Xaa Gln Arg
1 5

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What is claimed is:

1. A D-enzyme comprising an amino acid residue sequence that defines an polypeptide able to catalyze an enzymatic reaction, said amino acid residue sequence consisting essentially of D-amino acids.
- 5 2. The D-enzyme of claim 1 wherein said enzymatic reaction has achiral substrate specificity.
3. The D-enzyme of claim 2 wherein said enzyme is superoxide dismutase or carbonic anhydrase.
- 10 4. The D-enzyme of claim 1 wherein said enzymatic reaction has chiral substrate specificity.
5. The D-enzyme of claim 4 wherein said enzyme is selected from the group consisting of HIV-1 protease, D- [Aba^{67,95,167,195}]HIV-1 protease (HIV-1 PR), and D- [Aba^{67,95}(CO-S)^{51,52}]HIV-1 protease analog.
- 15 6. A method of producing a chirally pure chemical comprising:
 - a) reacting in an aqueous admixture a first stereoisomer with a D-enzyme that specifically convert said first stereoisomer into a chiral reaction product, said reacting occurring for a time period and under reaction conditions sufficient to form said reaction product, wherein said D-enzyme comprises an amino acid residue sequence that defines an enzyme able to catalyze said reaction, and said amino acid residue sequence consists essentially of D-amino acids; and
 - 20 b) isolating said chiral reaction product from said admixture, thereby forming said chirally pure chemical.
- 25 7. The method of claim 6 wherein said aqueous admixture comprises a racemic mixture having at least a first and a second stereoisomer.
- 30 8. A method of producing a chirally pure chemical comprising:
 - a) reacting in an aqueous admixture a racemic mixture having at least a first and a second

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stereoisomer with a D-enzyme that specifically converts said first stereoisomer into a reaction product, said reacting occurring for a time period and under reaction conditions sufficient to convert
5 substantially all of said first stereoisomer into said reaction product, wherein said D-enzyme comprises an amino acid residue sequence that defines an enzyme able to catalyze said reaction, and said amino acid residue sequence consists essentially of
10 D-amino acids; and

b) isolating said second stereoisomer from said admixture, thereby forming said chirally pure chemical.

9. A method for catalyzing a reaction involving
15 an achiral substrate comprising the following steps:

Step A: providing a D-enzyme having a specificity for the achiral substrate; then
Step B: contacting the achiral substrate with the D-enzyme of said Step A to catalyze the
20 reaction involving the achiral substrate.

10. A method for catalyzing a reaction as described in claim 9, wherein:

in said Step A: the D-enzyme is D-superoxide dismutase and the achiral substrate is a superoxide radical; and

25 in said Step B: the D-superoxide dismutase of said Step A is contacted with superoxide radical to convert to the superoxide radical to molecular oxygen and hydrogen peroxide.

30 11. A crystal comprising co-crystallized D- and L-enantiomers of an enzyme.

12. A method of structurally analyzing an L-enzyme comprising the following steps:

Step A: providing a crystal comprising co-crystallized D- and L-enantiomers of the L-enzyme; then

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Step B: exposing the crystal of said Step A to X-rays for generating an X-ray pattern; and then

5 Step C: analyzing the X-ray pattern generated in said Step B for structural information relating to the L-enzyme.

13. A method for screening a library for drug candidates having activity with respect to an L-enzyme, the method comprising the following steps:

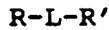
10 Step A: providing a D- enzyme, the D- enzyme being an enantiomer of the L- enzyme; then

Step B: screening the library to identify one or more elements of the library having activity in connection with the D- enzyme; and then

15 Step C: identifying drug candidates with respect to the L- enzyme using one or more of the elements of the library identified in said Step B.

14. A method as described in claim 13 wherein the L- enzyme includes a receptor or binding site.

15. A ligated D-protein represented by the formula:



25 wherein R and R' are D-peptides and L represents a linkage unit for ligating R to R'.

16. A ligated D-protein as described in claim 15 having in excess of 100 D-amino acid residues.

30 17. A ligated D-protein as described in claim 15 wherein L is a thiol ester or selenol ester linkage.

1 / 11

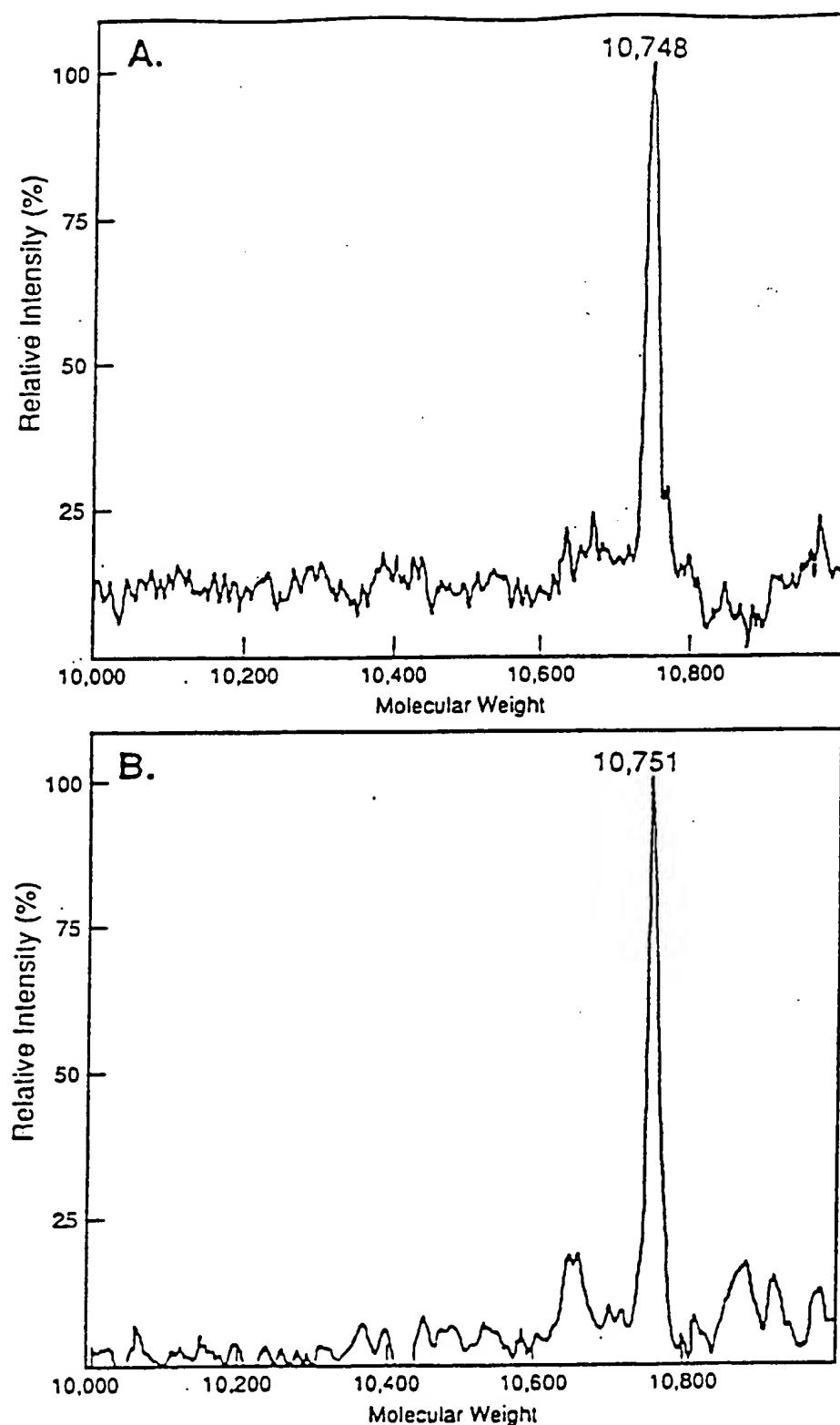


FIGURE 1

2 / 11

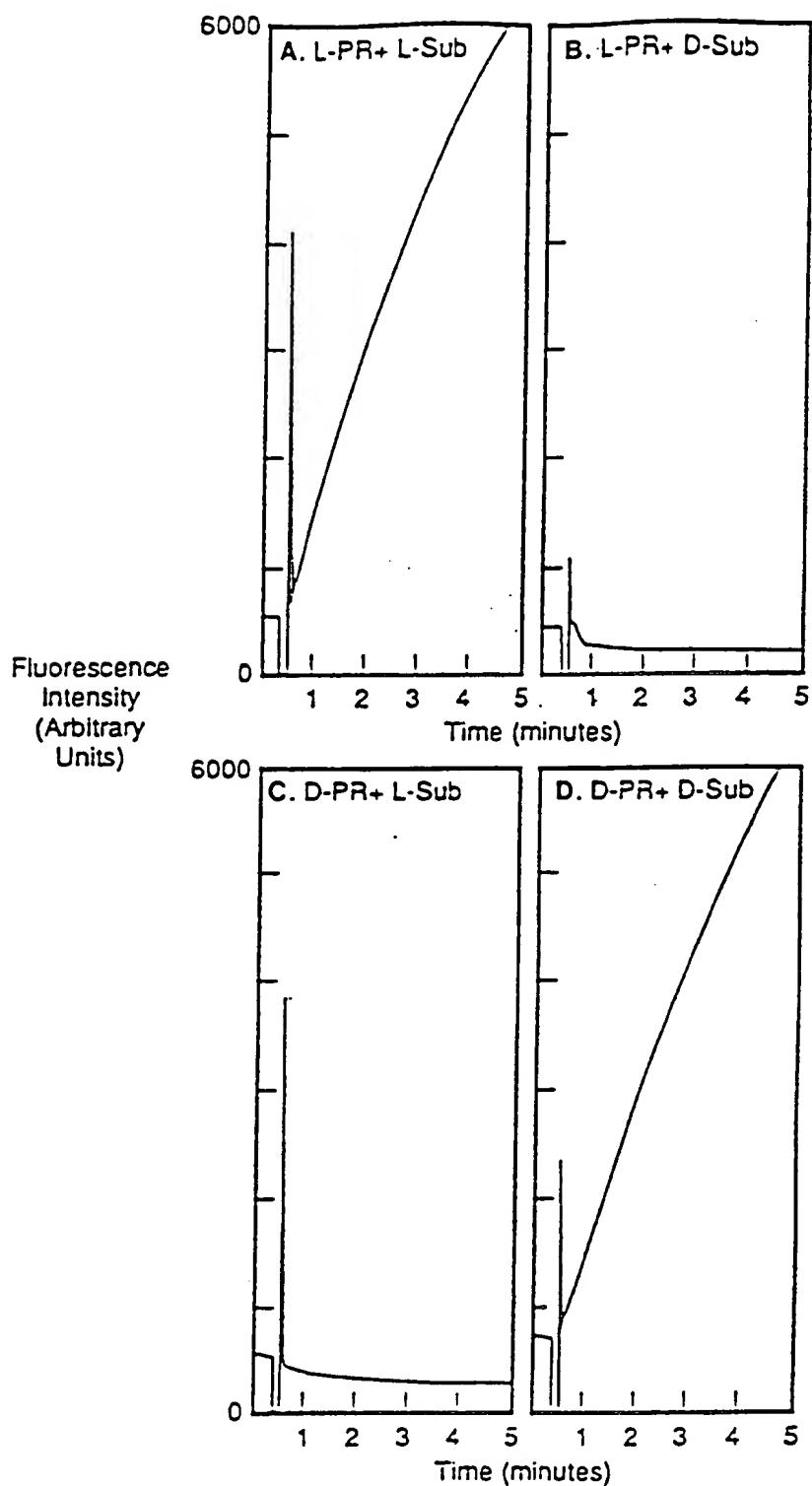
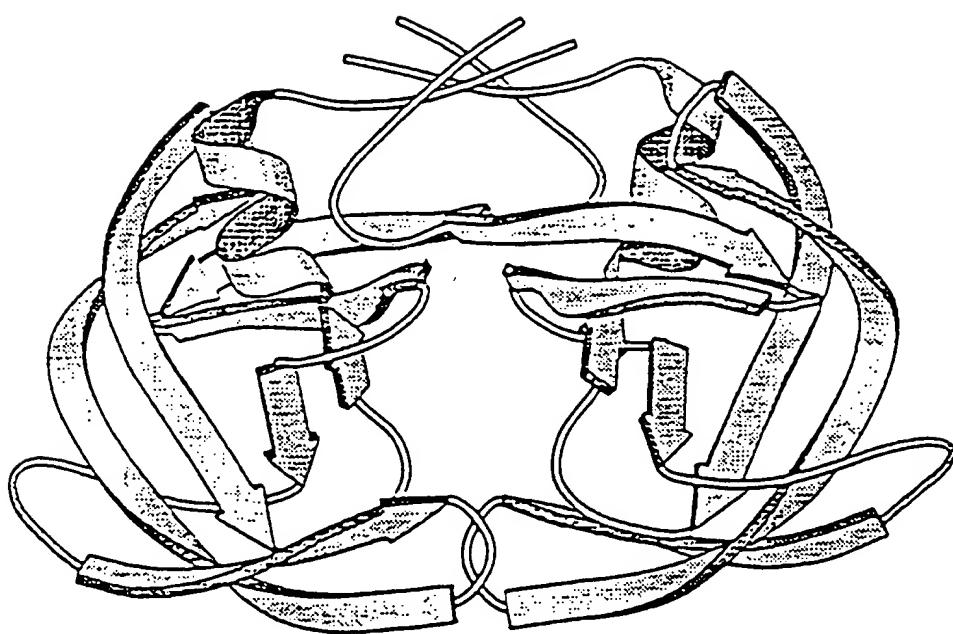


FIGURE 2

3 / 11

D-HIV PROTEASE



L-HIV PROTEASE

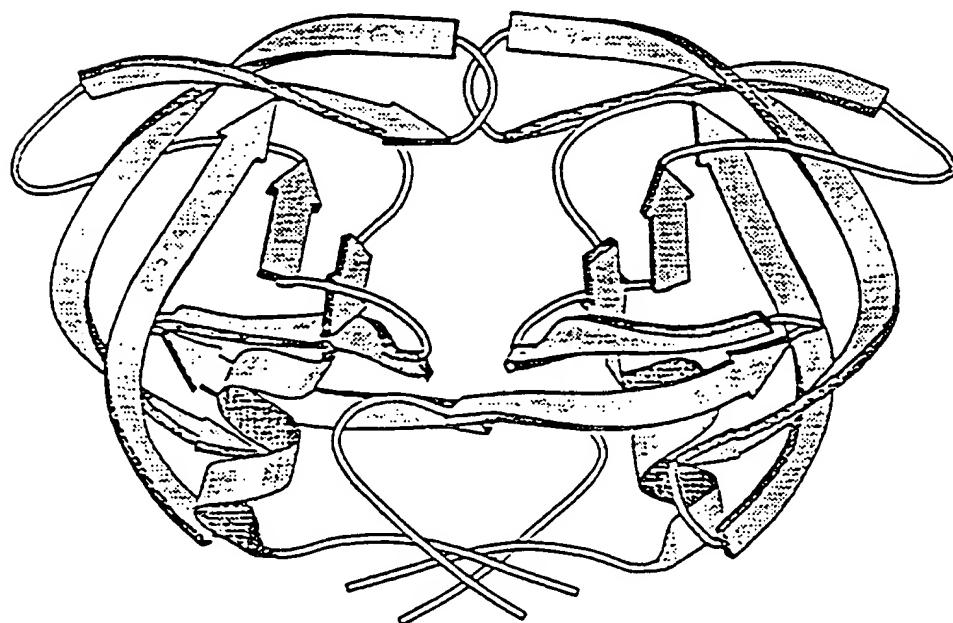


FIGURE 3

4 / 11

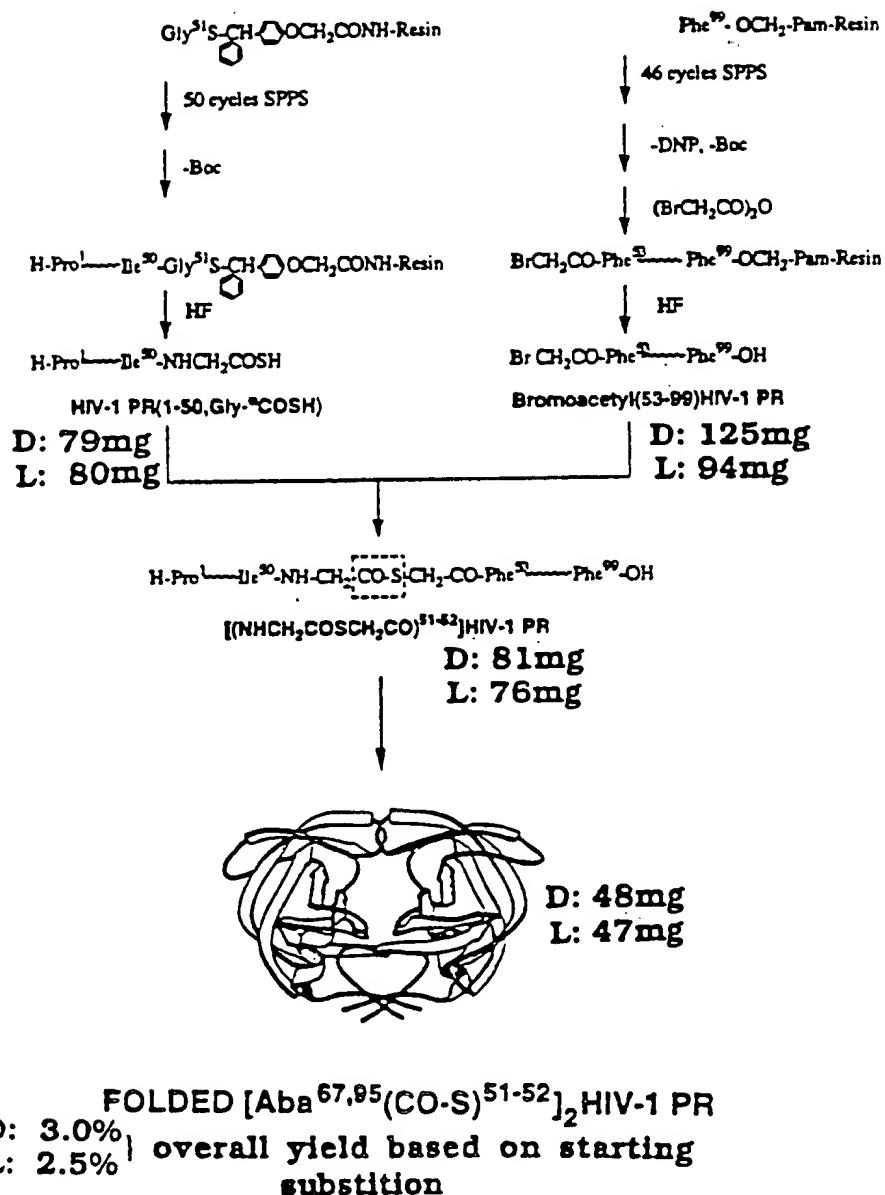
SCALE: 0.2 mmole

FIGURE 4

5 / 11

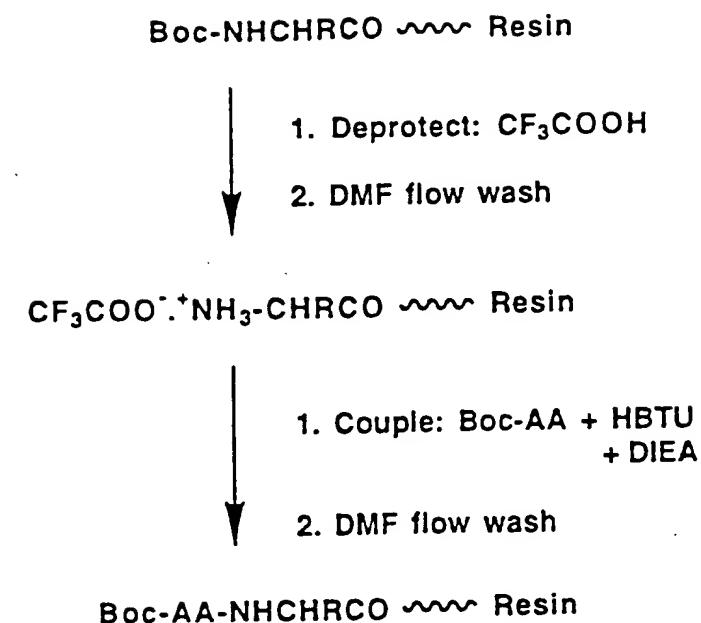


FIGURE 5

6 / 11

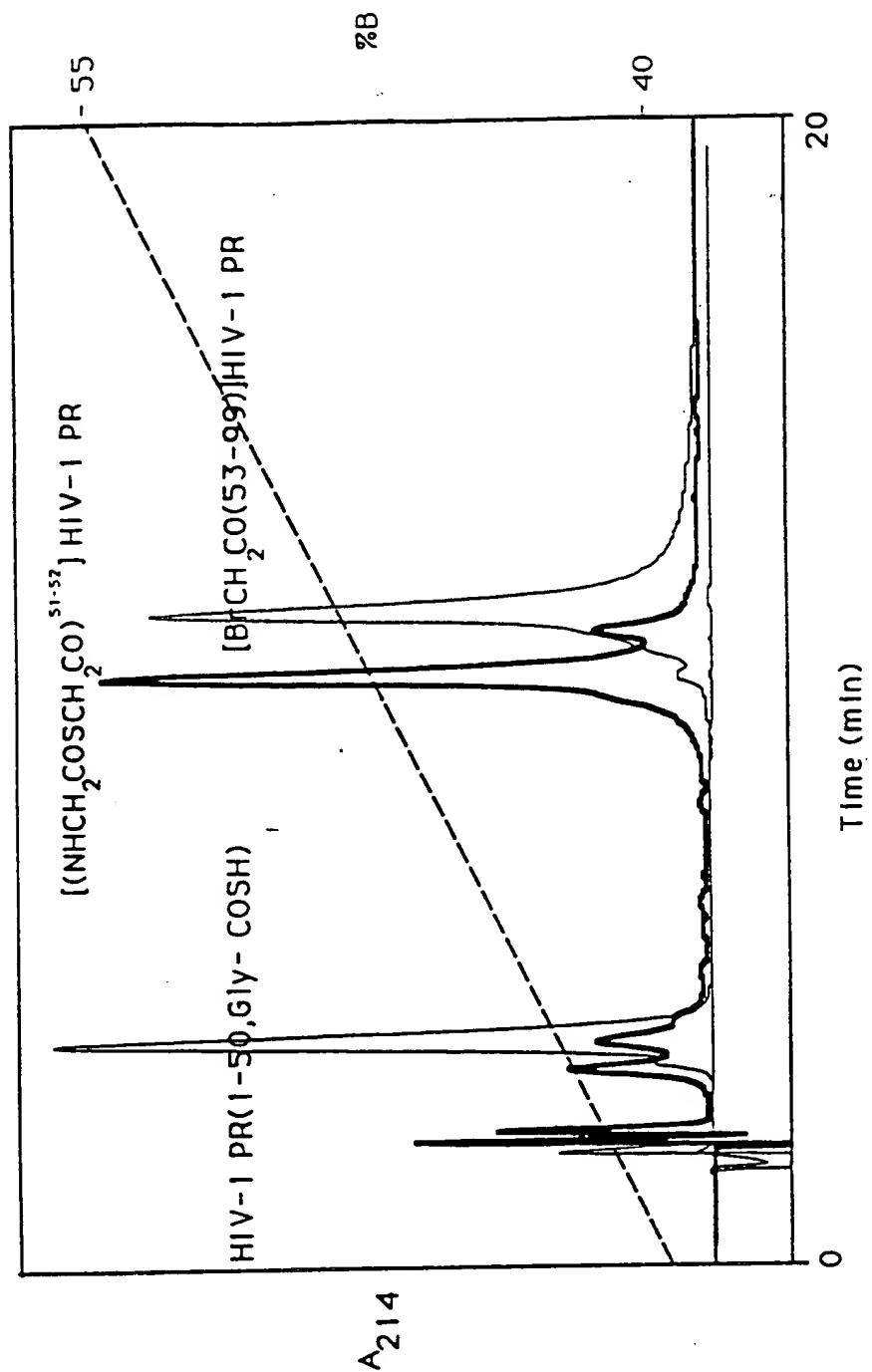


FIGURE 6

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	L		D	
	mg	%	mg	%
HIV-1 PR (1-50, Gly ^α -COSH)	80.1	5.0	79.0	4.4
Bromoacetyl (53-99) HIV-1 PR	93.6	7.6	124.6	9.3
[$(\text{NHCH}_2\text{COSCH}_2\text{CO})^{51-52}$] HIV-1 PR	75.8	67.4	80.8	44.0
Folded [Aba ^{67,95} , (CO-S) ⁵¹⁻⁵²] ₂ HIV-1 PR	47.4*	53.3	48.2*	60.0
OVERALL YIELD		3.0		2.5

*Yield extrapolated from a folding experiment with a small amount of material

FIGURE 7

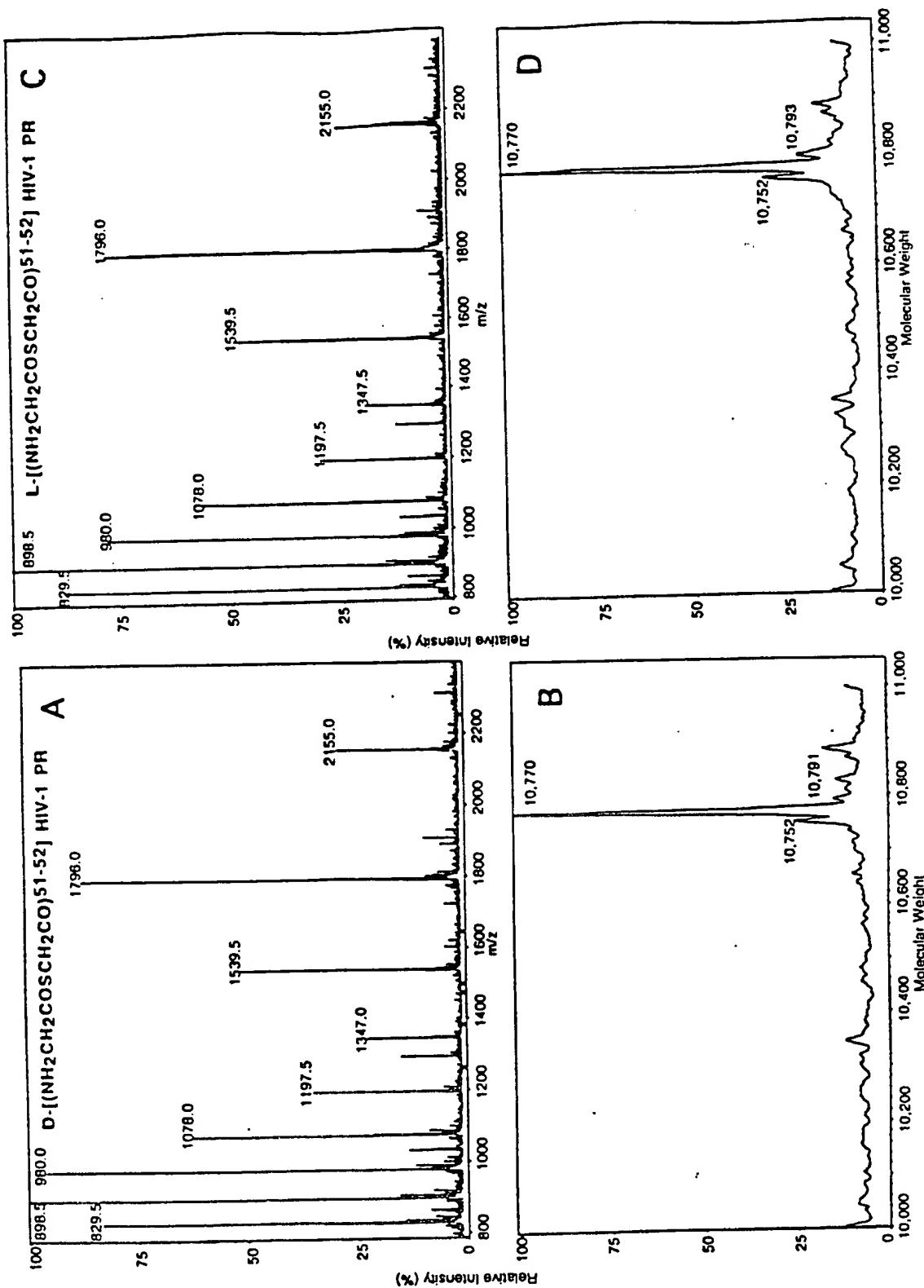


FIGURE 8

9 / 11

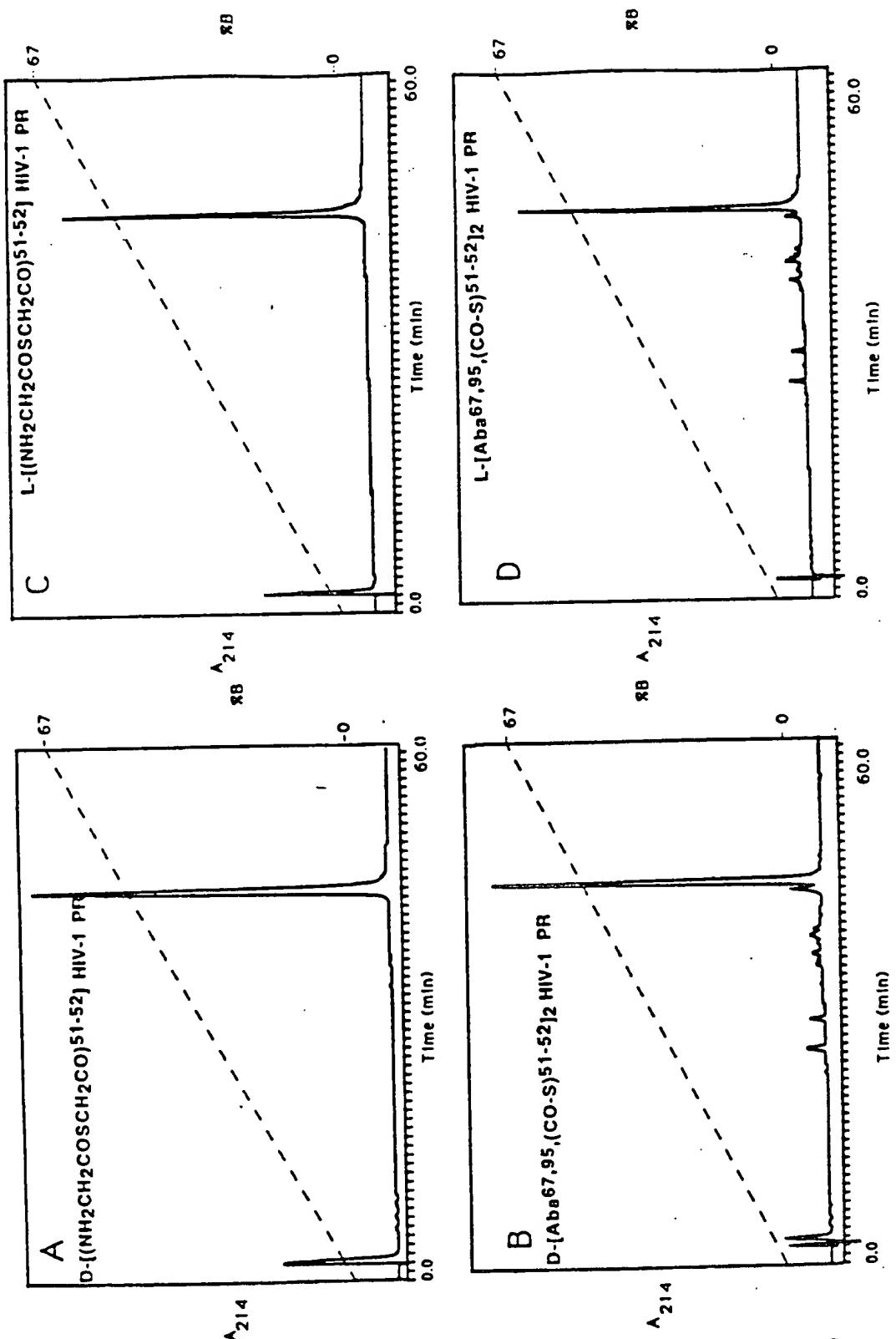


FIGURE 9

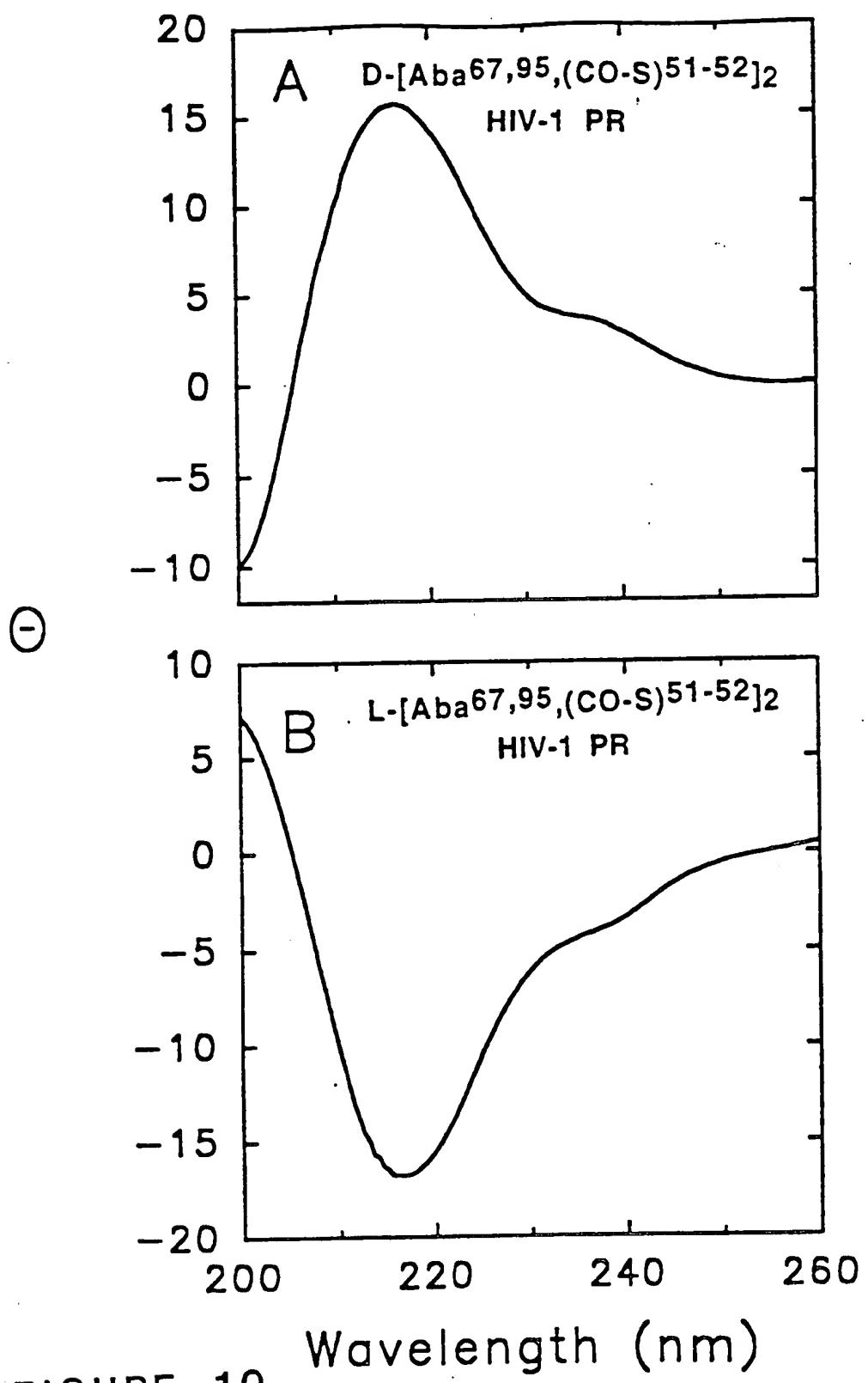


FIGURE 10

11 / 11

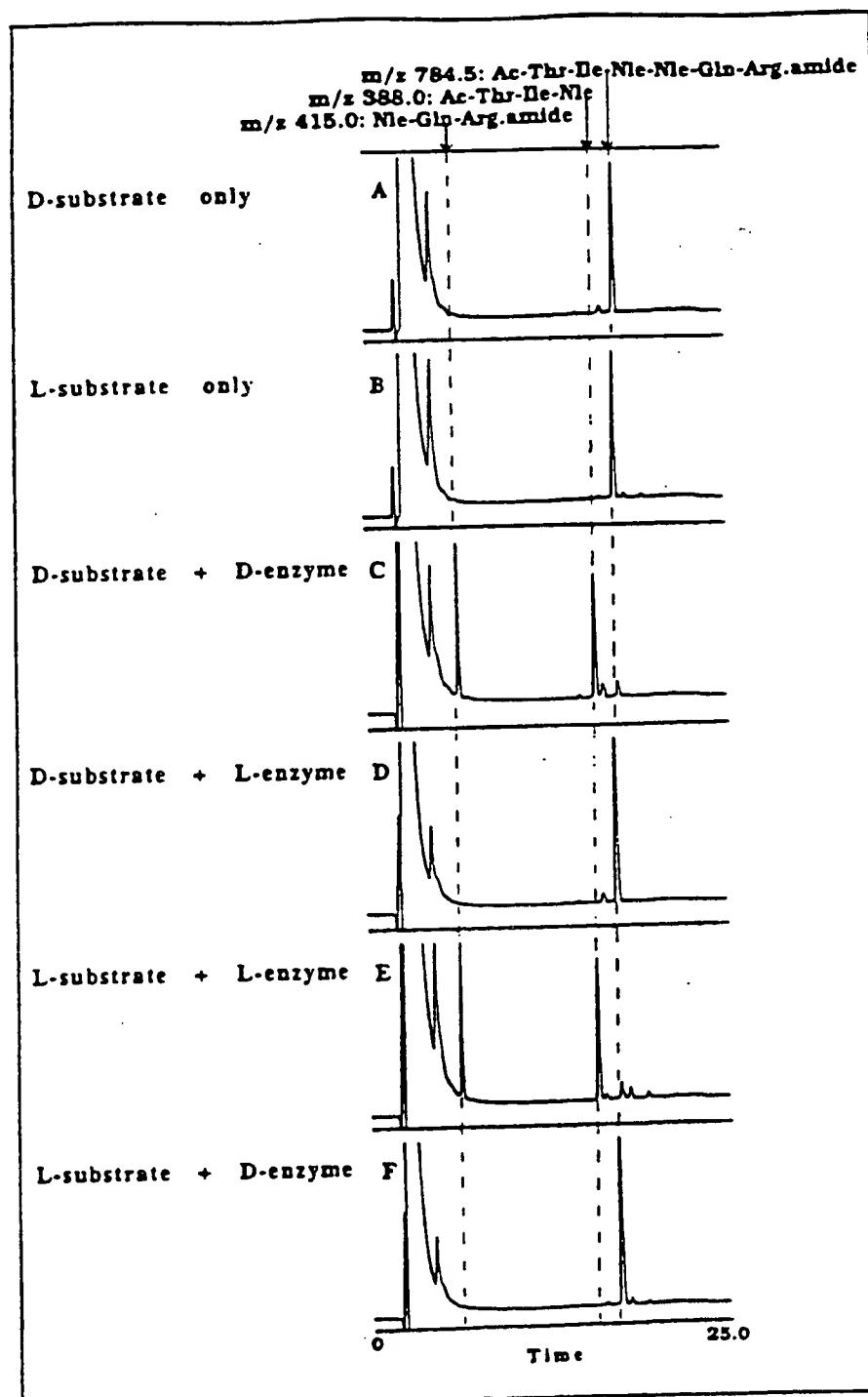


FIGURE 11

INTERNATIONAL SEARCH REPORT

Int'l	Application No
PCT/US 93/05441	

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5	C12N9/00	C12N9/02	C12N9/88	C12N9/50	C12P41/00
	C12P3/00	C12P13/00			

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 12 , June 1990 , WASHINGTON US pages 4761 - 4765 DAVID WADE ET AL. 'All-D amino acid-containing channel-forming antibiotic peptides' see abstract see page 4761, left column, paragraph 2 - right column, paragraph 1</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

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Date of the actual completion of the international search

27 October 1993

Date of mailing of the international search report

12-11-1993

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+ 31-70) 340-3016

Authorized officer

MONTERO LOPEZ, B

INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 93/05441

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SCIENCE. vol. 256 , 10 April 1992 , LANCASTER, PA US pages 221 - 225 MARTINA SCHNÖLZER ET AL. 'Constructing proteins by dovetailing unprotected synthetic peptides: Backbone-engineered HIV protease' cited in the application see abstract see page 224, middle column, paragraph 3 ---</p>	1,4,5, 15-17
P,X	<p>SCIENCE vol. 256 , 5 June 1992 , LANCASTER, PA US pages 1445 - 1448 R.C. DEL. MILTON ET AL. 'Total chemical synthesis of a D-enzyme: The enantiomers of HIV-1 protease show demonstration of reciprocal chiral substrate specificity' see abstract see page 1446, left column, paragraph 2 - middle column, paragraph 1 see page 1446, middle column, paragraph 4 ---</p>	1,4,5
P,X	<p>CHEMTRACTS: BIOCHEMISTRY AND MOLECULAR BIOLOGY vol. 3, no. 4 , July 1992 pages 211 - 213 CHARLES C. BIGELOW 'Total chemical synthesis of a D-enzyme: The enantiomers of HIV-1 protease show demonstration of reciprocal chiral substrate specificity' see page 211, paragraph 4 see page 212, paragraph 3 see page 212, paragraph 7 -----</p>	1,4,5

**Amendment No. 1 to Bilateral Confidential Disclosure Agreement between
Geron Corporation
and
Procter & Gamble Company**

This Amendment No. 1 ("Amendment No. 1") to the Bilateral Confidential Disclosure Agreement (the "Agreement") by and between Geron Corporation, having a principal place of business at 230 Constitution Drive, Menlo Park, California 94025 ("Geron"), and Procter & Gamble Company ("P&G"), is made effective 10 May, 2002 ("Effective Date").

Recitals

WHEREAS, Geron and P&G entered into that certain Bilateral Confidential Disclosure Agreement effective 10 May, 2001, with respect to technical and business information considered confidential by each party (the "Agreement"); and

WHEREAS, Geron and P&G now wish to amend the Agreement in accordance with this Amendment No. 1, as set forth herein.

NOW THEREFORE, P&G and Geron agree as follows:

1. The term of the Agreement as set forth in Section III of the Agreement, is hereby extended for one (1) year, up to and including 10 May, 2003.

All other terms and conditions of the Agreement shall remain unchanged and in full force and effect.

IN WITNESS WHEREOF duly authorized representatives of the Parties have entered into this Amendment as of the Effective Date first written above.

Geron Corporation

Procter & Gamble Company

By: _____
(signature)

By _____
(signature)

William D. Stempel

David N. DiGiulio

Title Vice President and General Counsel

Title Vice President, R&D
Global Personal Beauty Care

Date _____

Date _____

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Geron Corporation

Procter & Gamble Company

By: _____
(signature)

By _____
(signature)

William D. Stempel

David N. DiGiulio

Title Vice President and General Counsel

Title Vice President, R&D
Global Personal Beauty Care

Date _____

Date _____